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Chritian, Viral, 2001, “*Enzymes of Lignin-Degrading Fungi: Degradation of Xenobiotic Compounds*”, thesis PhD, Saurashtra University

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A thesis

Submitted for the

Degree of

Doctor of *Philosophy*

In

Microbiology

**ENZYMES OF LIGNIN-DEGRADING FUNGI:
DEGRADATION OF XENOBIOTIC COMPOUNDS**

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March, 2001

*.....to the name that is above every name;
in humble thanksgiving for the loving kindness and mercy,
which follows me through the days of my life.*

*“....but GOD is the strength of my heart
and my portion forever.”
- Psalms 73:26*

ACKNOWLEDGEMENTS

I would like to celebrate the completion of this thesis by acknowledging the help and support of many people who have made this possible and to whom I am indebted.

Let me begin by thanking my supervisor, Dr. Bharatkumar Rajiv Manuel Vyas, an exemplary scholar, an extraordinary guide, and more than being a guide, a make-feel-home friend; whose evident pleasure and deep interest in all my efforts and ideas, and determination to meet each one of us on the ground of our own being has been such an inspiration and morale. I appreciate his care and thoughtfulness with which he tested and extended my own understanding of this work,

I am grateful to the Head of the Bioscience Department Prof. S. P. Singh, and former Head of the Department Prof. P. P. Sood for providing me the laboratory and other facilities. Important to mention amongst the many others at the Department of Bioscience, Saurashtra University who have welcomed me in their company and taught me about the pleasures and pitfalls of academics are Dr. S. J. Pathak and Dr. S. C. Bhatt. The list is too long to mention; I am thankful to the entire non-teaching staff.

I gratefully acknowledge the Council of Scientific and Industrial Research (CSIR) for the Senior Research Fellowship and the Department of Special Assistance for the Junior Research Fellowship, which have re-instilled and affirmed my interests in working ahead.

Thanks to all my loving friends in helping me understand what I have achieved and how much better it could become, for always being there and making the lonely Rajkot a memorable event in my life. Rohit Shrivastava, Sudipta Misra and Dharmendra Shukla, for their friendship has been a source of continuity and delight to me; Dr. Nagar, who has been instrumental in leading me to Saurashtra University and has been my first guru amongst the woods of Barda and Gir; and Rupal Joshi and Mital Dodia, who have made each day a Saturday night for me.

I extend my deepest gratitude to my loving parents, Mr. Vinod and Mrs. Vinodini Christian, without whose unending love and support, I would never have reached to the place where I am; and to my parents-in-law, Mr. Navin and Mrs. Varsha Oza, for their ongoing interest and leaps of faith in me.

For Mrunal, my best friend and life-partner, for so much of togetherness from far away, for her enthusiasm and interest in my work, and for the genuine support she has offered during all the times; I feel deep affection and pride. I thank her for her integrity, forbearance and understanding as I disappeared mostly to Rajkot and so often into the solitary process of reading, writing and reflection.

I dedicate this work to my Lord and Savior, Christ Jesus.

(Viral Christian)

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CHAPTER 1

(A) PHYSIOLOGY AND BIOCHEMISTRY OF LIGNIN

DEGRADTION BY WHITE-ROT FUNGI

The term “Lignin” is derived from the Latin word “Lignum” meaning wood. Lignin is one of the most abundant biopolymer and aromatic material. Lignin probably rivals cellulose, the most abundant renewable organic resource, in reduced carbon and photosynthetic energy content. It occurs with cellulose in the form of lignocellulosic material and makes up a substantial fraction of the total mass of the biosphere. Thus, it plays a significant role in the carbon cycle (Crawford, 1981).

LIGNIN BIOSYNTHESIS

Lignins are complex racemic aromatic heteropolymers derived mainly from three hydroxycinnamyl alcohol monomers differing in their degree of methoxylation: p-hydroxycinnamyl (coumaryl) alcohol, which give rise to p-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxy-cinnamyl (coniferyl) alcohol, the guaiacyl units; 3,5-dimethoxy-4-hydroxycinnamyl (sinappyl) alcohol, the syringyl units (Fig. 1a.1). Most gymnosperm lignins contain primarily guaiacyl units and angiosperm lignins contain equal amounts of guaiacyl and syringyl units, whereas both contain only small amounts of p-hydroxyphenyl units.

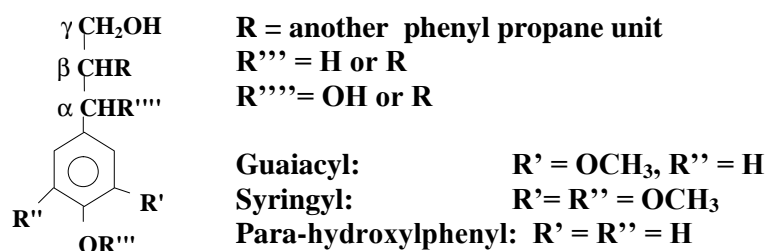


Figure 1a.1 Lignin monomers

Lignification is the process by which units are linked together via radical coupling reactions. The main “end-wise” reaction couples a new monomer to the growing polymer, giving rise to the lignin structure. Lignin precursors are formed by different enzymes system (Fig. 1a.2). Lignin is formed through dehydrogenative polymerization of the monolignols.

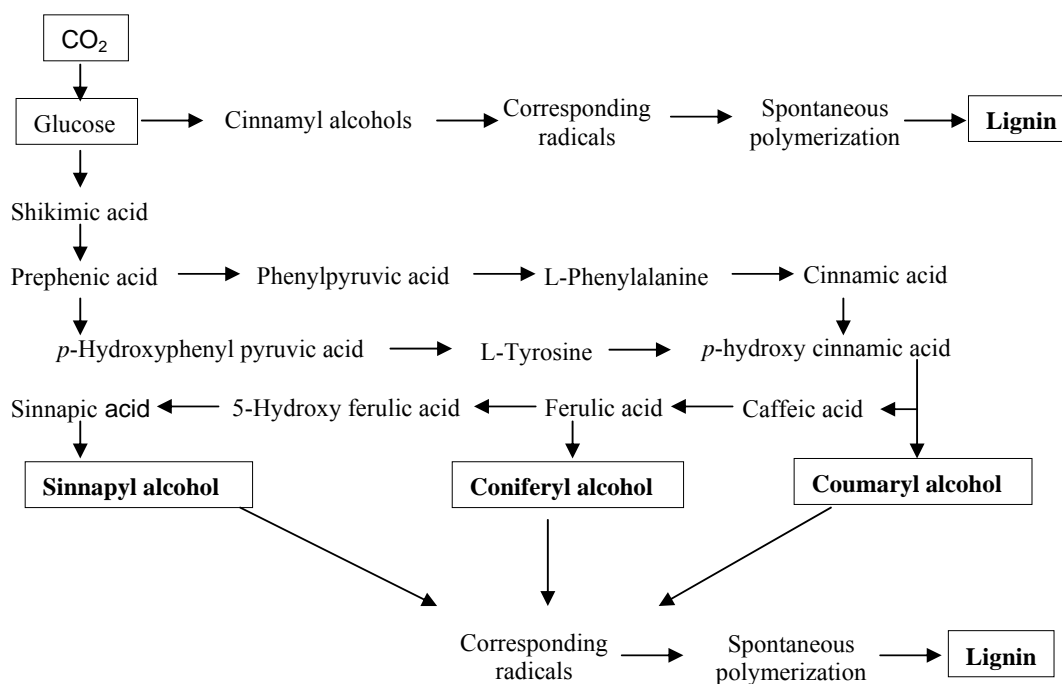


Figure 1a.2 Lignin biosynthesis pathway

LIGNIN STRUCTURE

Lignin is a heterogeneous, water-insoluble, optically inactive, random and highly branched three dimensional polymer. Lignin is racemic and amorphous. It contains several different interunit linkages, many of which are non-hydrolysable and biologically stable carbon to carbon and ether linkages.

Stereoirregularity of lignin makes it resistant to attack by enzymes for degradation. Further complexity in lignin is introduced by rearrangement of certain of the structures formed by radical coupling and presence of chiral carbon in both D and L form. It contains several interunit linkages, many of which are non-hydrolysable and biologically stable carbon to carbon and ether linkages.

LIGNIN DEGRADATION

In nature, there is a degradation of dead plant material mainly lignocellulosics by saprophytic microorganisms continually, efficiently and on tremendous scale. The only organisms known to degrade lignin more or less extensively are the wood rotting fungi (Kirk and Farrell, 1987). They can be divided into three groups according to the type of the decay they cause in wood.

Soft rot fungi

Soft rot fungi belong mainly to the Ascomycetes and Deuteromycetes. This type of rot is characterized by a softening of wood tissue accompanied by significant weight loss. The soft rot fungi degrade cellulose and hemicellulose, but only little lignin. They penetrate the secondary wall of the wood cell, forming cylindrical cavities in which the hyphae propagate. Soft-rot fungi cause demethylation of lignin and have got limited ability to degrade the side chains and aromatic rings. The rot is of limited extent, being closely associated with the fungal hyphae, because the cellulase enzymes do not diffuse freely through the wood (Deacon, 1983).

Brown rot fungi

Brown rot fungi are members of the Basidiomycetes and mainly degrade cellulose and hemicellulose, leaving the lignin more or less intact as a brown layer. Demethylation is the most obvious consequence of attack on lignin by these fungi. They introduce -carbonyl group into the propane side chains of the monomers. Brown rot fungi cause a generalized, diffuse rot by growing mainly in the cell lumen next to the secondary wall. They tend to cause an irregular decay pattern in the wood (Deacon, 1983).

White rot fungi

White rot fungi include several members of the Basidiomycetes from the Hymenomycetes, Agaricales and Aphyllophorales groups, but also some of the Ascomycetes, such as the *Xylaria* and *Ustilina* spp. Fungi of this group are able to degrade all the major components of wood and are generally considered to be the main agents of lignin decomposition in nature. These fungi have various patterns of colonization of the wood, but when present in the cell lumen they cause a progressive decay of the wall layers producing a progressive thinning of the walls (Deacon, 1983). There are several species of fungi being studied for their lignin-degrading capacity, such

as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, *Phlebia radiata*, *Ceriporiopsis subvermispora* and *Bjerkandera adusta*.

White rot basidiomycetous fungi make up the only group of microorganisms known to completely degrade and mineralize lignin (Zabell and Morell, 1992). Since lignin's carbon content is 50% higher than that of the polysaccharides, making relatively more abundant as a carbon / energy repository than its weight would indicate, the basidiomycetes therefore play a pivotal role in the biogeochemical cycling of carbon. Basidiomycetes are effective at these degradation processes because:

- They contain genes encoding extracellularly secreted lignin degrading enzymes.
- They are able to utilize substrate within a broad range of C / N ratio.
- The filamentous nature of their growth generates mechanical pressure that allows them to efficiently invade both soil and wood.
- Their ability to translocate nutrients throughout their mycelial thallus enables them to grow through nutritionally deficient zones in soils to seek out suitable substrates.

Ligninolysis is attributed to the secretion of peroxidases, H₂O₂-generating oxidases, and organic acids as a part of ligninolytic system. These different components of ligninolytic system generate radicals that bring about non-specific oxidation of lignin polymer (Kirk and Farrell, 1987).

PHYSIOLOGY OF LIGNIN DEGRADATION

The nature and the mechanism of the ligninolytic system of the white-rot basidiomycetes have been largely elucidated. In the cultures of *P. chrysosporium*, ligninolytic activity appears after a considerable delay following nitrogen starvation; this activity is produced until the exhaustion of carbon source. When a nutrient (C, N or S, but not P) becomes limiting, fungi adapt to the limitation by complex regulatory controls that results in cessation of net replicative growth, and shift to “secondary” or “idiophasic” metabolism that accommodates the limitations. Lignin degradation is, therefore, a “secondary metabolic” or “idiophasic” event. The white-rot fungi do not use lignin as a sole carbon and energy source and only a small fraction of lignin carbon enters the central metabolic pathway that is not enough to support the growth of the fungus. The onset of ligninolytic

activity following nitrogen depletion occurs simultaneously with the beginning of synthesis of a secondary metabolite, veratryl alcohol. The metabolite is synthesized from phenylalanine via 3, 4-dimethoxycinnamyl alcohol and veratryl glycerol. Repression by glutamate indicated that the nitrogen addition cause a biochemical repression of secondary metabolism. Glutamate stops not only the oxidation of lignin to CO₂ but also its depolymerization, apparently entire ligninolytic system is regulated as a part of secondary metabolism. High concentration of oxygen in culture fluids hastens up the appearance of ligninolytic activity and also increases its titre. Culture parameters that support lignin degradation and growth of the fungi are quite different e.g. pH, temperature, etc.

BIOCHEMISTRY OF LIGNIN DEGRADATION

Lignin polymer metabolism entails oxidative reactions is logically expected from the virtual nonhydrolyzability of lignin. Practical implications, of the oxidative nature of lignin degradation include, direct participation of molecular oxygen indicated by ¹⁸O₂ experiments (Trojanowski *et al.*, 1984). Non-specificity is evidenced by the facts that: a) lignins of different compositions are degraded, including those of grasses, hardwoods and conifers; b) lignin is degraded despite the heterogeneity of interunit linkages and the variety of neighbouring groups around those linkages; c) it is degraded despite the racemic nature of all asymmetric carbon; d) it is degraded even after substantial modification by chemical pulping and bleaching reactions (Lundquist *et al.*, 1977); and e) ligninolytic cultures metabolise a wide variety of aromatic compounds.

LIGNIN DEGRADING ENZYMES

Ligninolytic system of white rot basidiomycetes consists of a pool of enzymes, namely laccases, lignin peroxidase, manganese peroxidase, versatile peroxidase, cellobiose dehydrogenase, H₂O₂-producing enzymes and reactive oxygen species produced by such enzymes.

Laccases

Laccases (EC 1.10.3.2) are extracellular glycoproteins having molecular mass of 60-80 kDa with 15-20% carbohydrate content. Laccases are blue copper oxidases that catalyse the one-electron oxidation of phenolics and other electron-rich substrates. Most ligninolytic fungi produce laccases, *P. chrysosporium* being a notable exception. Laccases

contain multiple copper atoms which are reduced as the substrates are oxidized. After four electrons have been received by a laccase molecule, the laccase reduces molecular oxygen to water, returning to the native state. Laccases have very broad substrate specificity with respect to the electron donor. They catalyze the removal of a hydrogen atom from the hydroxyl group of *ortho*- and *para*-substituted mono and polyphenolic substrates and from aromatic amines by one-electron abstraction to form free radicals capable of undergoing further depolymerization, repolymerization, demethylation, or quinone formation (Thurston, 1994). Oxidation of methoxyhydroquinones during lignin degradation followed by autooxidation of the resulting methoxysemiquinones results in the formation of superoxide anion radicals, which can undergo further reactions (Guillen *et al.*, 2000).

Unique, so-called “yellow laccases” have been discovered in cultures of *Panus tigrinus* and *Phlebia radiata*. They have been demonstrated to oxidize nonphenolic lignin models and veratryl alcohol in the absence of any diffusible mediator (Leontievsky *et al.*, 1997). Recently, a novel hydrogen peroxide-dependent phenol oxidase (TAP) from *Termitomyces albuminosus* was able to oxidize various phenolic compounds but not veratryl alcohol (Johjima *et al.*, 2003). The rather broad substrate specificity of laccases may be additionally expanded by addition of redox mediators, such as ABTS, 1-hydroxybenzotriazole, or compounds secreted by ligninolytic fungi, but it is not yet known whether natural versions of such auxiliary substrates function *in vivo* in lignin biodegradation (Bourbonnais and Paice, 1992), and indeed, the actual role of laccase has yet to be fully clarified.

Lignin peroxidase (LiP)

Lignin peroxidase was (EC 1.11.14) the first ligninolytic enzyme to be discovered (Glenn *et al.*, 1983; Tien and Kirk, 1983). The enzyme is a glycoprotein that contains about 15% carbohydrates and an iron protoporphyrin IX (heme) as a prosthetic group. It has a molecular weight of 41-42kDa and a pH optimum of 2, but the enzyme is unstable at this low pH (Tien, 1987). The lignin peroxidase family contains multiple isoenzymes. (Kirk and Farrell, 1987). The catalytic cycle of lignin peroxidase is shown in Figure 1a.3. The resting enzyme reacts with H₂O₂ to produce the two-electron oxidized intermediate, Compound I i.e. Feryl -porphyrin cation radical, for which veratryl alcohol (VA) acts as an electron mediator and get converted to veratryl alcohol cation radical that in turn

oxidizes the lignin substrate to yield the one-electron oxidized intermediate Compound II. Compound II returns to the resting enzyme by oxidizing a second VA molecule. The free radical can undergo a variety of reactions. With excess H_2O_2 Compound II can be transformed to Compound III, oxyperoxide, which is an inactive form of the enzyme (Tien 1987).

VA enhances the action of LiP on many substrates, including lignin (Hammel *et al.*, 1993), by acting as a mediator (Harvey *et al.*, 1986), or by protecting the enzyme against inactivation (Wariishi and Gold, 1989). Veratryl alcohol is oxidized via cation radical intermediates, which are known to be powerful charge-transfer reagents that can oxidize large hydrophobic molecules like lignin. LiP catalyzed the conversion of VA to veratryl aldehyde, with the enzyme-bound VA cation radical as an intermediate that in turn can oxidize other recalcitrant molecules by indirect oxidation. LiP catalyses the oxidative depolymerization of polymeric lignin *in vitro* (Hammel *et al.*, 1993). Lignin peroxidase can oxidize both phenolic and non-phenolic lignin related compounds resulting in cleavage of the C - C β bond, the aryl C bond, aromatic ring opening, phenolic oxidation and demethoxylation.

LiP catalyzed reduction

A mechanism has been elucidated whereby LiP can catalyze reductions using veratryl alcohol (Barr *et al.*, 1992). The veratryl alcohol cation radical readily reacts with oxalate to produce the carboxylate anion radical and reduced back to veratryl alcohol. The carboxylate anion radical is a powerful reducing agent. In the absence of another electron acceptor, the carboxylate anion radical will reduce molecular oxygen to the superoxide anion radical, which in turn reduces ferric iron to ferrous iron. Hydrogen peroxide then readily reacts with chelated ferrous iron to produce hydroxyl radical (Barr *et al.*, 1992).

Manganese peroxidase (MnP)

Manganese peroxidase (EC 1.11.13) is also a heme-containing glycoprotein and it forms a family of isoenzymes, was discovered in *P. chrysosporium* (Glenn and Gold, 1985; Paszczynski *et al.*, 1985). The molecular weight is approximately 46 kDa (Kuwahara *et al.*, 1984). The catalytic cycle of MnP is essentially the same as for LiP with the exception that Mn(II) is necessary to complete the cycle. As shown in Figure 1a.4, the native enzyme reacts with H_2O_2 forming Compound I, which is then converted to

Compound II by reacting with one equivalent of Mn(II) and forming Mn(III). A second Mn(II) is then used to reduce Compound II back to the native enzyme.

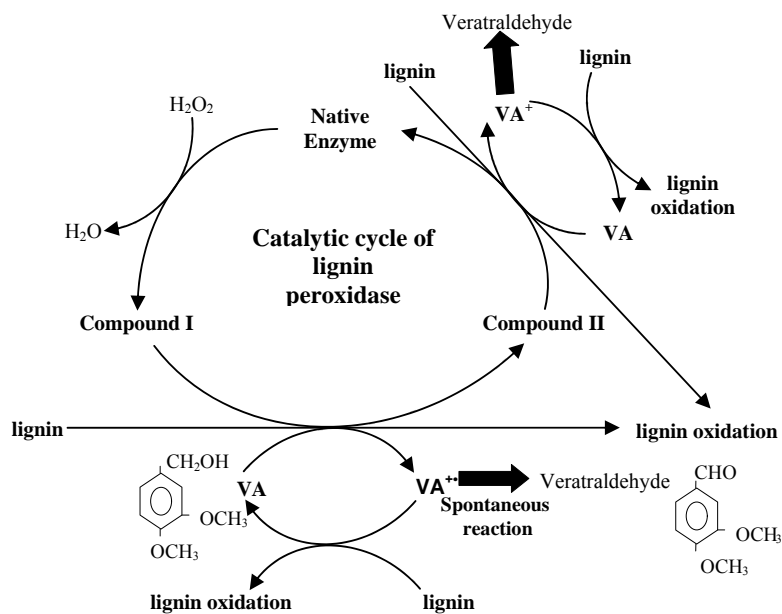


Figure 1a.3 Catalytic cycle of lignin peroxidase

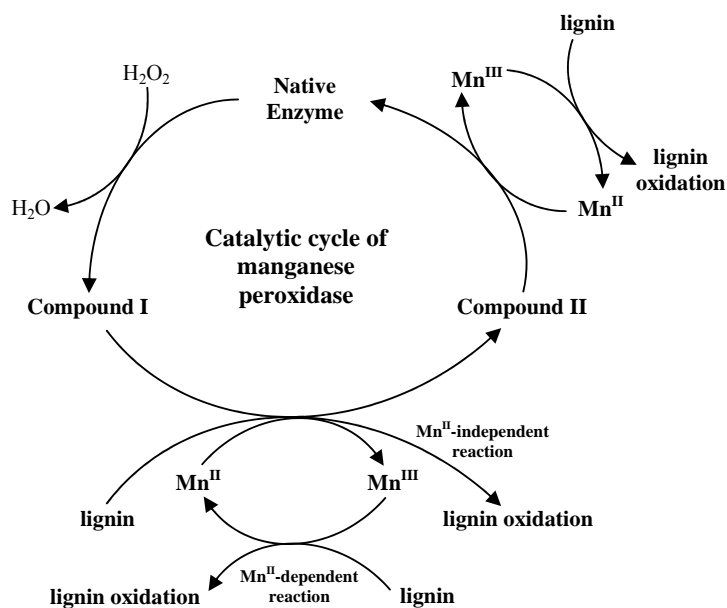


Figure 1a.4 Catalytic cycle of manganese peroxidase

Similarly, the MnP Compound I can be reduced by phenolic substrates, but at a slower rate. Phenolic compounds are, however, not able to reduce efficiently MnP Compound II back to the native enzyme. It may be that the FeIV=O center in MnP Compound II is partially buried and the site is not available to organic substrates. Thus, the enzyme is not able to complete its catalytic cycle without the presence of Mn(II) (Wariishi *et al.*, 1988).

The presence of α -hydroxy acids such as oxalate activates the system by chelating Mn(III) to form stable complexes. These complexes oxidize several of the substrates of MnP. The enzyme also has oxidase activity, producing hydrogen peroxide by oxidation of reduced substrates like NAD(P)H, glutathione, dithiothreitol and dihydroxymaleic acid (Paszczyński *et al.*, 1988). Phenolic and amino-aromatic compounds are oxidized by hydrogen abstraction to form phenoxyl and amino radicals, respectively (Glenn and Gold, 1985). Chelates of Mn^{3+} and carboxylic acids can react with each other and are converted to alkyl radicals, which undergo subsequent spontaneous reactions with dioxygen resulting in the formation of superoxide radicals (Hatakka, 2001).

These radicals are thought to be a source of peroxides that are generated via autocatalytical reactions and can be used by MnP in the absence of external H_2O_2 (Hofrichter *et al.*, 1998). Certain co-oxidants such as organic sulphur compounds (e.g. glutathione-GSH, L-cystein, etc.) as well as unsaturated fatty acids and their derivatives (e.g. linoleic acid, Tween 80) are oxidized by the MnP system to form particularly reactive thiyl and peroxy radicals, respectively (Kapich *et al.*, 1999). In the presence of dioxygen, these radicals can attack recalcitrant lignin structures, which are normally not open to attack by the “simple” MnP system (Kawai *et al.*, 1995) and they are also a source of H_2O_2 (Paszczyński *et al.*, 1985).

MnP oxidizes various monomeric and dimeric phenols, including phenolic lignin model compounds. The underlying mechanism is based on an initial one-electron oxidation of the substrate by enzyme-generated Mn^{3+} , which produces a phenoxy radical intermediate. This radical is further oxidized by Mn^{3+} to form a carbon-centered cation. Subsequent loss of a proton yields the ketone dimer, whereas an attack by water on cation, which is followed by alkyl-phenyl cleavage of the arylglycerol-aryl structure, produces other products. However, alternative mechanisms are involved for oxidation of nonphenolic structures. Lipid peroxidation generates reactive intermediates (peroxy radicals) and

brings about the degradation of nonphenolic lignin dimers (Kapich *et al.*, 1999). The MnP system is a potent biochemical tool, acting as a “radical pump”, to attack the recalcitrant lignin polymer.

Cellobiose dehydrogenase (CDH)

The CDH (EC 1.1.99.18) is a flavohemoprotein with a molecular weight of 90 kDa. CDH carries two prosthetic groups: namely, an FAD and heme in two different domains. The FAD-containing domain carries all known catalytic and cellulose binding properties. Non-heme portion of CDH has been found in the culture filtrate of some fungi and was for a long time believed to represent a separate enzyme, cellobiose:quinone oxidoreductase (CBQ). CDH supports lignin degradation by reducing aromatic radicals, generated by ligninolytic enzymes (Temp and Eggert, 1999). It also reduces toxic quinones to phenols that can be used as redox mediators by ligninolytic enzymes (Morpeth, 1991). It supports MnP in three different ways: a) dissolves precipitated Mn(IV)O₂ by reduction; b) produces cellobionic acid by spontaneous hydrolysis of produced cellobionolactone, which complex Mn(III), and c) reduces quinones (Roy *et al.*, 1994). CDH also reduces compound II of ligninolytic peroxidases and thus complete the catalytic cycle in the absence of peroxidase substrate (Ander *et al.*, 1993). It degrades and modifies cellulose, hemicellulose and lignin by generating hydroxyl radicals in Fenton type reaction (Kremer and Wood, 1992a).

CDHs reportedly can use electrons from the oxidation of cello-oligosaccharides (Kremer and Wood, 1992b) to reduce free radicals to phenolics (Roy and Archibald, 1993), quinones (Westermarck and Eriksson, 1974), Fe (III) (Kremer and Wood, 1992a), Mn(III) (Bao *et al.*, 1993), and Mn(IV) (Roy *et al.*, 1994). Thus, CDH-mediated reduction produces many lignin-based structures that are good substrates for the laccases and MnPs commonly secreted by white rot fungi during delignification.

Pyranose 2-dehydrogenase

A novel C-2 specific sugar oxidoreductase, designated as pyranose 2-dehydrogenase having molecular weight of 79 kDa, has been isolated and characterized (Volc *et al.*, 1996). Using 1,2-benzoquinone as an electron acceptor, pyranose 2-dehydrogenase oxidizes D-glucose to D-arabino-2-hexosulose (2-dehydroglucose, 2-ketoglucose). 3,5-di-tert-butyl-1,2-benzoquinone, 2,6-dichlorophenol-indophenol, and ferricyanide also serve

as pyranose 2-dehydrogenase electron acceptors. Pyranose 2-dehydrogenase plays an important role in fungal lignocellulose decomposition by interconnecting ligninolysis with degradation of cell-wall polysaccharide components. This quinone-reducing enzyme is likely to be involved in further breakdown of toxic monomeric quinone intermediates generated during extracellular peroxidative oxidation of lignin (Volc *et al.*, 1996).

Peroxide producing enzymes

Following the discovery of lignin peroxidases, the search for enzymatic sources for hydrogen peroxide production resulted in the discovery of extracellular and intracellular oxidases in ligninolytic cultures of white-rot fungi, producing H₂O₂ from glucose (Eriksson *et al.*, 1986), methanol, glyoxal (Kersten and Kirk, 1987) and related compounds. Furthermore, MnP produces H₂O₂ from reduced NADH, NADPH and certain thiol-containing compounds (Glenn and Gold, 1985).

Glucose oxidase

Glucose oxidase (EC 1.1.3.4) possesses two functions necessary for acceleration of lignocellulose breakdown: by means of oxidation of glucose it produces hydrogen peroxide which is necessary for peroxidase activity. It also reduces quinones and phenoxy radicals yielded by laccase during oxidation of lignin. It cooperates with the system of cellulases oxidizing glucose generated by these enzymes during hydrolysis of cellulose (Leonowicz *et al.*, 1997).

Laccase oxidizes phenol-derived radicals to quinones, which serve as the oxygen source for glucose oxidase (Leonowicz *et al.*, 1999). On the other hand, excess quinones produced by laccase inhibit the enzyme (Szklarz and Leonowicz, 1986), suggested that glucose oxidase counteracting the poisonous level of quinones in the medium enables laccase to continue its function. Glucose oxidase cooperates with LP and MnP, providing hydrogen peroxide and reducing quinones yielded by laccase to adequate phenols (Szklarz and Leonowicz, 1986; Leonowicz *et al.*, 1999). It acts as a regulating enzyme in transformation of lignocelluloses and operates as a feedback system.

Glyoxal oxidase (GLOX)

Hydrogen peroxide is also produced by the extracellular enzyme Glyoxal oxidase (EC 1.2.3.5). Substrates for GLOX are methylglyoxal, glycoaldehyde, acetaldehyde,

formaldehyde, glyoxal, glyoxylic acid, dihydroxyacetone and glyceraldehydes at pH 6 (Kersten and Kirk, 1987). C-C cleavage of arylglycerol-aryl ethers by LiP gives glycoaldehyde and further oxidation by GLOX produces 3 molecules of H₂O₂. GLOX also has some veratryl alcohol oxidase (VAO) and veratryldehyde oxidase activity (Hammel *et al.*, 1994). Thus, the intimate cooperation of lignin peroxidases and GLOX may lead to controlled radical formation and limitation of radical coupling, driving lignin oxidation towards depolymerization (Kurek and Kersten, 1995).

Pyranose oxidase

The enzyme pyranose oxidase (P2O) (EC 1.1.3.10), which catalyzes the oxidation of several aldopyranoses to yield the corresponding 2-ketoaldoses, is widely distributed among wood-degrading basidiomycetes (Leitner *et al.*, 1998). Typically, P2O is rather large, homotetrameric protein that contains covalently bound FAD. The *in vivo* substrates of P2O are D-glucose, D-galactose, and D-xylose, which are abundant in lignocellulose and which are oxidized to 2-keto-D-glucose, 2-keto-D-galactose and 2-keto-D-xylose, respectively. In addition, P2O also exhibits significant activity with a number of other carbohydrates, including L-sorbose, D-glucosno-1, 5-lactone, and D-allose (Freimund *et al.*, 1998). During the oxidation reactions electrons are transferred to molecular oxygen, resulting in the formation of hydrogen peroxide (Giffhorn, 2000). P2O is localized primarily in the periplasmic space. Only during autolysis, it is located extracellularly. Hydrogen peroxide produced by P2O in the periplasmic space of fungal hyphae or extracellularly may function *in situ* with two lignin-degrading enzymes, LP and MnP, for which H₂O₂ is an essential substrate.

Aryl alcohol oxidases (AAO)

Aryl alcohol oxidases (EC 1.1.3.7) are having narrower substrate specificity. AAO from *Phanerochaete chrysosporium* is produced in high nitrogen medium during primary metabolism and is also the only AAO reported to be inducible by aryl alcohols (e.g. vanilyl alcohol). Veratryl alcohol oxidases (VAOs) are flavoproteins containing one molecule of FAD per molecule of protein. This coenzyme acts as a redox centre in the transfer of two hydrogen atoms from aryl alcohols to molecular oxygen. VAO have wide substrate specificity towards differently substituted methoxy phenols. The physiological role of this enzyme has been related to the ligninolytic activity of white-rot fungi because of hydrogen peroxide production during the reductive half reaction. Guillen and Evans,

(1994) proposed that VAO and intracellular NADPH-dependent aryl alcohol dehydrogenase could constitute a redox system involving aryl alcohols / aryl aldehydes produced in order to ensure a steady concentration of hydrogen peroxide suitable for ligninolytic activities. VAO catalyze the oxidation of aryl - and -unsaturated - alcohols to the corresponding aldehydes with concomitant reduction of O₂ to H₂O₂.

Reactive oxygen species (ROS)

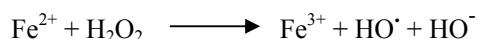
Lignocellulose-degrading enzymes are too large to penetrate lignified cell walls in sound wood (Blanchette *et al.*, 1996). Research has refocused on ROS such as hydroxyl radicals ([•]OH), peroxy radicals (ROO[•]) or hydroperoxyl radicals ([•]OOH), which might be the agents that initiate fungal decay within the secondary wood cell wall.

Hydroxyl radical

The hydroxyl radicals ([•]OH) have received more attention than any other ROS in studies of wood decay. [•]OH is a reasonable candidate for wood decay agent because it is the strongest oxidant that can occur in aqueous systems. It reacts rapidly with virtually all organic molecules, either by abstracting hydrogens from aliphatic structures or by adding as an electrophile to aromatic ones (Halliwell and Gutteridge, 1999). [•]OH abstracts hydrogen atoms from the sugar subunits of polysaccharides such as cellulose and produces transient carbon-centered radicals that react rapidly with oxygen to give ROO[•] species. If the peroxy radical already carries a hydroxyl group on the same carbon, it eliminates [•]OOH (Halliwell and Gutteridge, 1999). Studies with lignin model compounds indicate that [•]OH attacks the subunits of lignin (Ek *et al.*, 1989), both by abstracting aliphatic C -hydrogens and by adding to aromatic rings. When hydrogen abstraction occurs, the product is a benzylic ketone, whereas addition to the ring gives a hydroxylated cyclohexadienyl radical. Under acidic conditions, this ring oxidized intermediate can combine with a proton and eliminate water, thus yielding an aryl cation radical that undergoes carbon-carbon bond cleavage and other degradative reactions (Gierer, 1990).

Production of Fenton's reagent

Almost hundred years ago, Fenton discovered the strong oxidizing power of mixture of hydrogen peroxide and Fe (II), which is abundant in wood (Fenton, 1894). The active species was later shown to be the hydroxyl radical, formed by what has become known as the Fenton reaction.



·OH from CDH

CDH can act as a cellobiose oxidase, thus reducing O_2 and producing H_2O_2 , but Fe^{3+} is a better electron acceptor than O_2 , and as a result, CDH can act as Fe^{3+} reductases (Henriksson *et al.*, 2000). CDH system is a good generator of Fenton reagent in wood. White-rot fungi and brown-rot fungi secrete oxalic acid which is a strong chelator of Fe^{3+} and Fe^{2+} . CDH can reduce Fe^{3+} -oxalate and Fe^{3+} -trioxalate complex to Fe^{2+} or Fe^{2+} -monooxalate complex effectively which is stable at pH 2.5, but as they diffuse away from hyphae into a region with a lower oxalate conc, they will encounter a higher pH, which will result in formation of Fe^{2+} -dioxalate complex. Around pH 4, Fe^{2+} -dioxalate complexes autooxidize the oxalate dianion, and as a result $\cdot OHH$ is produced. This $\cdot OHH$ is reduced by Fe^{2+} or dismutates, thus generating H_2O_2 , the second ingredient needed for Fenton chemistry (Hyde and Wood, 1997).

·OH from quinone redox cycling

The principle of this mechanism is that the fungus reduces an extracellular quinone to its hydroquinone, which then reacts with Fe^{3+} to give Fe^{2+} and semiquinone radical. The semiquinone then reduces O_2 to give $\cdot OHH$ and the original quinone. Because $\cdot OHH$ is a source of H_2O_2 ; this cycle will generate a complete Fenton system (Kerem *et al.*, 1999). Enzymes potentially capable of this reaction include intracellular benzoquinone reductases from *P. chrysosporium* (Brock *et al.*, 1995). Other possible quinone reducing enzymes include extracellular sugar dehydrogenases such as CDH, which have been shown to use quinones as alternate electron acceptor (Henriksson *et al.*, 2000).

Other ·OH generating system

Some of the extracellular phenolic compounds secreted by fungus carry enough reducing equivalents that each of them can reduce multiple molecules of Fe^{3+} (Goodell *et al.*, 1997). Another mechanism is that wide variety of wood decay fungi produce extracellular Fe^{2+} -binding glycopeptides that could play role in Fenton chemistry (Tanaka *et al.*, 1999).

Peroxyl and hydroperoxyl radicals

If wood decay fungi produce $\cdot OH$, then it is also necessary to consider the effects that $ROO\cdot$ and $\cdot OHH$ have on lignocellulose, because both of these ROS are expected as secondary radicals when $\cdot OH$ oxidizes wood polymers. In addition, white rot fungi produce extracellular lipid that could serve as a source of peroxyl radicals (Enoki *et al.*,

1999). The manganese-dependent peroxidases of white rot fungi peroxidize unsaturated fatty acids, which results in the formation of ROS that include ROO \cdot (Moen and Hammel, 1994). The Mn $^{3+}$ chelates produced by MnP do not initiate lipid peroxidation by abstracting allylic hydrogens, but rather abstract a methylene hydrogen from the carbon adjacent to the fatty acid's carboxyl group. The resulting resonance-stabilized acyl radicals then propagate lipid peroxidation and ROO \cdot formation by abstracting allylic hydrogens from unsaturated fatty acids (Watanabe *et al.*, 2000).

Although ROO \cdot and \cdot OHH are less reactive than \cdot OH, they are strong oxidants, and consequently attack lignocellulose. They abstract benzylic hydrogens, such as those at C of the lignin side chain (Kapich *et al.*, 1999). It is likely that they can also abstract hydrogens from polysaccharides, because an adjacent hydroxyl or ether oxygen will stabilize the carbon-centered radical that is formed (O'Neal and Benson, 1973). Peroxyl radicals can also abstract electrons from aromatic ethers such as those found in lignin, and this reaction results in ligninolytic reactions similar to those that lignin peroxidase catalyzes. However, these electron transfer reactions are slow unless the attacking peroxyl radical contains electron-withdrawing substituents (Kapich *et al.*, 1999).

Other enzymes

Versatile peroxidase (VP)

LiP and MnP have been considered as two models for all ligninolytic peroxidases. A novel ligninolytic peroxidase recently described in *Pleurotus* and *Bjerkandera* species (Martinez *et al.*, 1996). VP combines the catalytic properties of both peroxidases, being able to oxidize typical MnP and LiP substrate. VP is able to perform both the oxidative reactions characteristic of *P. chrysosporium* LiP, i.e. the oxidation of nonphenolic aromatic substrates via aromatic radicals, and MnP, i.e. the oxidation of Mn $^{2+}$ to Mn $^{3+}$. *Pl. eryngii* peroxidase efficiently oxidizes substituted phenols, which cannot be oxidized by *P. chrysosporium* peroxidases. In addition to above substrate VP can oxidize two compounds, -keto- -methylthiobutyric acid and *p*-methoxybenzene (Kuwahara *et al.*, 1984). VP is able to oxidize Mn $^{2+}$ to Mn $^{3+}$, degrade the lignin model dimers veratrylglycerol- -guaiacyl ether yielding veratryldehyde, and oxidize veratryl alcohol and *p*-dimethoxybenzene to veratryldehyde and *p*-benzoquinone respectively..

RBBR oxygenase

Our group discovered that *Pleurotus ostreatus* produced an unusual enzyme along with the ligninolytic enzymes during solid-state fermentation of wheat straw that was capable of Remazol brilliant blue R (RBBR) decolorization (Vyas and Molitoris, 1995). This activity is independent of MnP, laccase and VAO also produced by the isolate, and from LiP activity which is not detectable in *P. ostreatus* but are well-known in other white-rot fungi. The activity was named RBBR oxygenase.

APPLICATION OF LIGNIN DEGRADATING SYSTEM

Lignocellulose conversion

Extant and potential uses of fungi for lignocellulose conversion can be divided into four categories: a) conversion into food or feed; b) manufacture of mechanical pulp; c) production of microbial products; d) treatment of lignocellulose derived wastes

Conversion into food or feed

The direct use of lignocellulosic residues as ruminant animal feed, or as a component of such feed, represents one of its oldest and widespread applications (Hadar *et al.*, 1992). The lignocellulose complex in straw and other plant residues is degraded very slowly because of the physical barrier imposed by lignin polymers, preventing free access of hydrolytic enzymes such as cellulases and hemicellulases to their substrates. The purpose of delignification is to increase the accessibility of polysaccharides to enzymatic hydrolysis. Substantial increases in crude protein have been reported for woods, barks and lignocellulosic wastes following cultivation of various white-rot fungi (Ek and Eriksson, 1980). Similarly, increases in *in vitro* polysaccharide digestibility of lignocelluloses have accompanied solid substrate incubation with white rot fungi (Zadrazil, 1980).

Manufacture of mechanical pulp

The knowledge that WRF do not cause a rapid depolymerization of cellulose as they decay their substrates and lower the lignin content of wood, has led to use of WRF as pulping agent and concept of “Biopulping”. Biopulping is defined as the treatment of wood chips with lignin degrading fungi prior to pulping. Possibility of partially delignifying wood by solid-state fermentation with WRF to reduce the energy requirement for mechanical pulping and to improve the strength properties of resulting pulp has been extensively perused. WRF alter the wood cell walls, which softens the

chips and substantially reduces the electrical energy needed for pulping. Results of several investigators suggest that biological delignification of wood leads to less energy input for thermomechanical pulping and gave pulps with good burst and tensile strength. The residual lignin in unbleached kraft pulp is degraded by WRF, increasing the brightness of the pulp.

Production of microbial products

Lignocellulose-degrading fungi produce a variety of products. Lignin solubilized by WRF might be used in adhesive formulations and as an immunoadjuvant (Crawford and Pometto, 1984). Enzymatic hydrolysis of lignocellulose can lead to ethanol production via saccharification (Duff and Murray, 1996). Several lignin-degrading fungi produce edible fruiting bodies (mushrooms) and can be used directly as food for humans. These saprophytic basidiomycetes have been successfully cultivated at a commercial level worldwide. The cultivation of edible fungi on animal manure and plant by products has many remarkable ecological advantages in food production. Because these fungi delignify their substrate as they grow, the material remaining after mushroom harvest is useful for animal feeding or enzymatic saccharification.

Treatment of lignocellulose derived wastes

The ability of the lignin degrading fungi to degrade cellulose, hemicelluloses and lignin that they might find utility in processes to treat wastes from lignocellulose-using industries. Lignin degrading fungi can be used for converting the waste liquors from sulphite pulping of wood (Forss *et al.*, 1977), converting waste water from fibreboard manufacture to a protein-rich feed (Ek and Eriksson, 1980), decolorize effluent from the chlorine bleach plants of kraft pulp mills (Fukuzumi *et al.*, 1977).

Bioremediation

Bioremediation can be defined as the use of biological processes for the cleaning of soil, surface waters and groundwater from contaminants. It has appeared as an interesting alternative to chemical and physical methods because biological processes have the potential to degrade completely the contaminants and not just transfer them from one phase or chemical structure to another. During the last few years bioremediation has emerged as an industry for cleaning of extensive areas of contaminated soils. Biological processes can be used not only to clean contaminated soils or waters as in bioremediation

(where a remedy is applied to a problem), but may also be used before contamination occurs as a mode of preventing further transport and spreading of the pollutants. In such cases, a more appropriate term is bioprophylaxis instead of bioremediation.

The non-specificity of ligninolytic enzymes permits WRF to degrade a wide range of structurally diverse compounds. Identification of the “brute-force” approach to lignin degradation of WRF led different research groups to test the ability of these fungi to degrade environmental pollutants (Barr and Aust, 1994; Novotny *et al.*, 1997; Vyas *et al.*, 1994a, b). Cultures of white rot fungi can degrade and mineralise polyaromatic hydrocarbons (PAH), (Vyas *et al.*, 1994a), organochlorines (Yadav and Reddy, 1993), dioxines (Gold *et al.*, 1992), nitroaromatics and explosives (Stahl and Aust, 1993), PCBs (Vyas *et al.*, 1994b), and dyes (Christian *et al.*, 2003, 2004; Shah and Nerud, 2002; Vyas and Molitoris, 1995).

The white rot fungi have been the subject of extensive investigation during the past years with the idea of applying the ligninolytic system to the degradation of xenobiotics. Mounting evidence suggests that the lignin degrading system is responsible, at least in part, for the unique biodegradative abilities of this fungus. Basidiomycetes constitute a promising group of microorganisms for application of bioremediation of contaminated sites (Novotny *et al.*, 2000).

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CHAPTER 1

(B) DEGRADATION OF XENOBIOTIC COMPOUNDS BY LIGNIN-DEGRADING WHITE-ROT FUNGI: ENZYMOLOGY AND MECHANISMS INVOLVED

The industrial development over the last five decades has resulted in an exponential increase in the production and consumption of chemicals. Production, use and disposal of numerous chemicals cause widespread contamination of soils as well as ground waters and surface waters. Indiscriminate applications, high persistence, unknown environmental pathway and pollutant's potential to bioaccumulate have resulted in severe repercussions, including the loss of food sources, mutagenic and carcinogenic effects to the mankind.

The recognition that environmental pollution is a worldwide threat to public health has given rise to a new industry for environmental restoration. Physical and chemical treatment processes (i) typically remove organic pollutants at low level, (ii) are highly selective in terms of the range of pollutants removed and (iii) prohibitively expensive for the treatment of wastes. Cleanup of environmental pollution also presents a serious economic burden and therefore cost effective yet efficient and environment-friendly methods of decontamination are vital in solving the hazardous waste problems. The use of indigenous or introduced microorganisms to decontaminate waste sites – bioremediation, provides a very attractive, eco-friendly and economic solution to many of our hazardous pollution problems. For both economic and ecological reasons, biological degradation has become an increasingly popular alternative for the treatment of hazardous wastes. One such method of bioremediation involves the white rot fungi, a group of basidiomycetes characterized by their ability to degrade lignin in wood. This degrading ability is unique among these fungi and has made them an important link in the global carbon cycle.

LIGNIN-DEGRADING WHITE ROT FUNGI

Most known white rot fungi (WRF) are basidiomycetes and are capable of white rot decay. White rot decay derives from the appearance of wood attacked by these fungi, in

which lignin removal results in a bleached appearance of the substrate. The ability to catabolize lignocellulose and hemicellulose is fairly common as a primary metabolic process among WRF. As a result, it is not regarded as a rate-limiting step in the carbon flux. Lignin is extremely recalcitrant and is mineralized in an obligatorily oxidative process, carried out appreciably only by white rot fungi (Zabell and Morrell, 1992). The oxidation of lignin yields no net energy gain, and so lignin is degraded during secondary metabolism in order to access wood polysaccharides locked in lignin-carbohydrate complex.

Ligninolytic activity appears during nutrient C, N, or S limitation. Under such conditions, white rot fungi produce certain extracellular enzymes viz. lignin peroxidase, manganese peroxidase, laccases, H₂O₂-generating enzymes, versatile peroxidase, cellobiose dehydrogenase, etc in various combinations. These enzymes are encoded by gene families that allow complex regulation and generation of isoforms, and catalyze one-electron oxidation resulting in the formation of reactive free radical species inside the lignin polymer. Subsequently, the radicals undergo spontaneous reactions leading to the incorporation of oxygen, bond cleavage and finally the breakdown of lignin molecule (Kirk and Farrell, 1987). The physiology and biochemistry of lignin degradation has recently been reviewed by our group (Christian *et al.*, 2004a). The unique ligninolytic enzyme system of basidiomycetous fungi would be ideal for the biodegradation of organopollutants in the environment (Fernando and Aust, 1994). Understanding the basic mechanism of lignin degradation is central to understanding how many highly oxidized environmental pollutants can be degraded by WRF.

Advantages of white-rot fungi

The white rot fungal technology is very different from other better-established methods of bioremediation. The mechanisms used by the fungi provide them with several advantages for pollutant degradation.

Many environmental pollutants are persistent because of their insolubility in water or bound in soil, which are only poorly accessible to bacteria because of their intracellular degradation machinery. The lignin-degrading system, being extracellular, has evolved to degrade insoluble chemicals such as lignin and many of hazardous environmental pollutants. Moreover, the extracellular system of WRF enables to tolerate and degrade

considerably higher concentration of toxic pollutants. In order to metabolize toxic pollutants, bacteria must take up the pollutant within the cell because of intracellular enzyme system, which in turn inhibit bacterial growth. The fact that the lignin-degrading system is non-specific, non-stereoselective and free radicals based in nature, may provide other advantages. That allows WRF to degrade a wide variety as well as complex mixtures of pollutants. Free radicals are highly reactive and reactions occur as chain reaction, carrying out complete degradation of pollutants. Whereas bacterial enzymes are highly specific and a consortium may be required to successfully and completely degrade such chemicals. The lignin-degrading system is expressed in response to nutrient (C, N or S) limitations and therefore organism does not require preconditioning with the pollutant to be degraded. Enzymes are not repressed even when the pollutant concentration is reduced to ineffective levels for enzyme induction. Bacteria cannot degrade the pollutant when the concentration is reduced below threshold value and ineffective for enzyme induction. WRF can effectively degrade very low concentrations of pollutants to non-detectable levels.

WRF can be cultivated on inexpensive growth substrates like wheat straw, corncobs, wood chips or other crop residues and also on liquid media as well as in soil that promote the use of WRF for bioremediation. In addition to being able to grow under nutrient limitation, the fungi also produce oxygen radicals such as the OH[•], which is capable of oxidizing biomolecules, such as proteins and DNA that could result in the death of other microbes. Using the plasma membrane dependent redox system, the fungus is able to adjust the pH of its surrounding environment. Thus, microbes with pH optimum that differ from that of the fungus might not grow well after the fungus has been introduced.

The extreme non-specificity of the mechanisms described here makes the WRF an attractive solution to many of our ever-growing hazardous waste problems. However, only through our understanding and continued research efforts, with regard to these mechanisms, will we be able to successfully design bioremediation strategies employing the WRF.

MECHANISMS FOR XENOBIOTIC DEGRADATION

(A) Lignin peroxidase

Direct oxidation

Lignin peroxidase (LiP) has a classical peroxidase catalytic mechanism. Native enzyme is oxidized by H_2O_2 and generates two electron deficient compound I. Compound I can oxidize a chemical and can be reduced to compound II which is one electron deficient. A subsequent oxidation of another molecule by compound II returns the peroxidase to its native resting stage (Fig. 1b.1). LiP is having relatively high redox potential (Kersten, 1990), so the chemicals with high redox potentials that are not oxidized by other enzymes are oxidized by LiP. LiP can oxidize both phenolic and non-phenolic compounds resulting in carbon-carbon bond cleavage, aromatic ring fission, phenolic oxidation, demethoxylation, methylation, hydroxylation and dimerization reactions same as with lignin (Gold *et al.*, 1989).

Many pollutants including benzo[a]fluorene, cyanides, dyes, etc. are directly oxidized by LiP *in vitro*. Cyanide was oxidized directly to cyanyl radical by LiP H2 isoenzyme. The ability of LiP to oxidize cyanide allows the fungus to efficiently degrade the pollutant to CO_2 (Shah and Aust, 1993). LiP also catalyzes direct oxidation of pyrene to pyrene-1,6-dione and pyrene-1,8-diones. Dibenzo[*p*]-dioxin is also oxidized by LiP to its cation radical (Hammel *et al.*, 1986). Various dyes have been found to be directly oxidized by LiP (Cripps *et al.*, 1990). Oxidation of methylene blue by LiP results into formation of Azure C, which is a tri-demethylated methylene blue derivative (Kling & Neto, 1991).

Indirect oxidation

In many cases, chemicals are not directly accessible to heme of LiP and thus direct oxidation does not occur. In such cases involvement of redox mediator plays an important role. Veratryl alcohol (VA) produced by WRF is an excellent substrate for LiP. VA serves as an electron mediator to facilitate oxidation of pollutants. VA is oxidized by LiP to VA cation radical ($\text{VA}^{*\cdot}$) which is a strong oxidant responsible for indirect oxidation of lignin and pollutants (Fig. 1b.2)

EDTA was found to be indirectly decarboxylated by LiP H2. The apparent inhibition of veratryl alcohol oxidase activity of LiP H2 by EDTA is suggestive of the reduction of $\text{VA}^{*\cdot}$ back to VA during oxidation of EDTA. (Shah *et al.*, 1992).

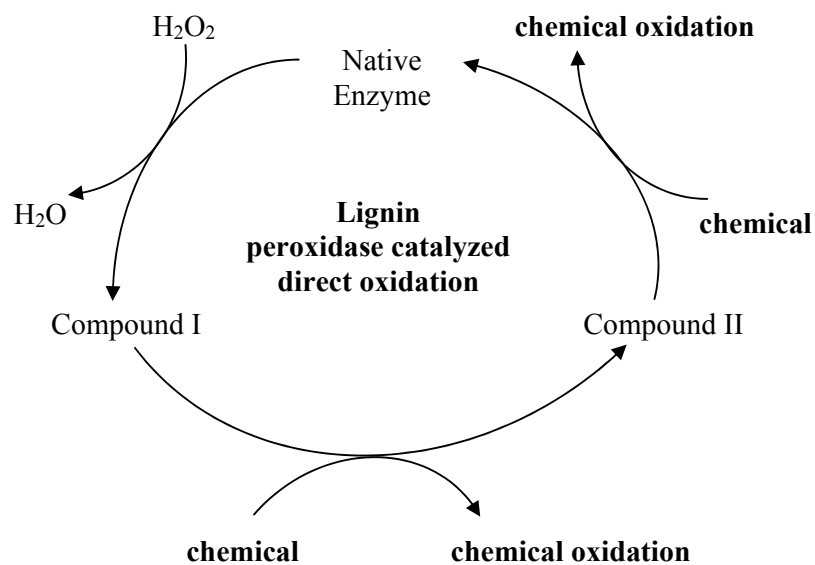


Figure 1b.1 Mechanism of direct oxidation by lignin peroxidase

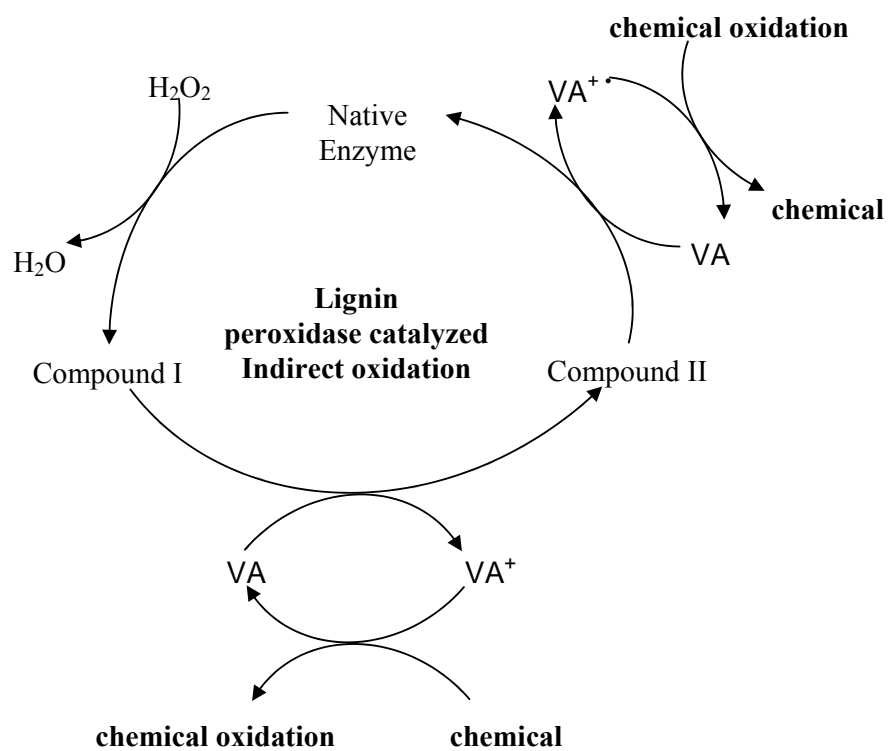


Figure 1b.2 Lignin peroxidase catalyzed indirect oxidation

Chemicals that have been found to be indirectly oxidized by LiP include herbicide aminotriazol (Tuisel *et al.*, 1992), pentachlorophenol (Chung and Aust, 1995a), phenol (Chung and Aust, 1995b), etc. Recently we reported direct as well as indirect oxidative decolorization of Remazol brilliant blue R by LiP produced by *Trametes versicolor* (Christian *et al.*, 2004b)

Reduction

LiP catalyzes reduction of various chemicals in the presence of VA (Fig. 1b.3). VA^{•+} generated in LiP reaction oxidizes carboxylic acids to respective acid derived anion radicals, which in turn serve as reductant. Such radicals effectively reduce cytochrome *c*, nitro blue tetrazolium, ferric ion and molecular oxygen and are also involved in the reduction of carbon tetrachloride to the trichloromethyl radical which is neither a substrate for enzyme nor a good reductant (Shah *et al.*, 1992).

VA^{•+} oxidizes EDTA as well as oxalate to their corresponding anion radicals. These carboxylate anion radicals, in the absence of another electron acceptor, reduce molecular oxygen to the O₂^{•-}, which will reduce ferric iron to ferrous iron (and has been shown to reduce some chemicals). H₂O₂ then readily reacts with chelated ferrous iron to produce [•]OH (Fig. 1b.3) (Barr *et al.*, 1992). [•]OH is having incredible oxidizing ability and make-up a potential non-enzymatic biological system known as Fenton reagent. Fenton's reaction has been widely used for degradation of xenobiotic compounds including PCBs, herbicides and dyes (Pratap *et al.*, 1998; Nerud *et al.*, 2001).

(B) Manganese peroxidase

Oxidation

MnP differs from LiP in that Mn²⁺ serves as the reducing agent of compound I and compound II generated upon subsequent oxidation of native enzyme. MnP oxidizes Mn²⁺ to Mn³⁺ which is stabilized by organic acid chelators and acts in turn as a low molecular mass, diffusible, redox mediator that attacks organic molecules and oxidizes various chemicals nonspecifically via hydrogen and one electron abstraction (Glenn and Gold, 1985) (Fig. 1b.4). Compound I besides Mn²⁺ can be reduced by other electron donors such as ferrocyanide and phenolics. Compound II is only very slowly reduced by other substrates and requires Mn²⁺ to complete the catalytic cycle (Wariishi *et al.*, 1988).

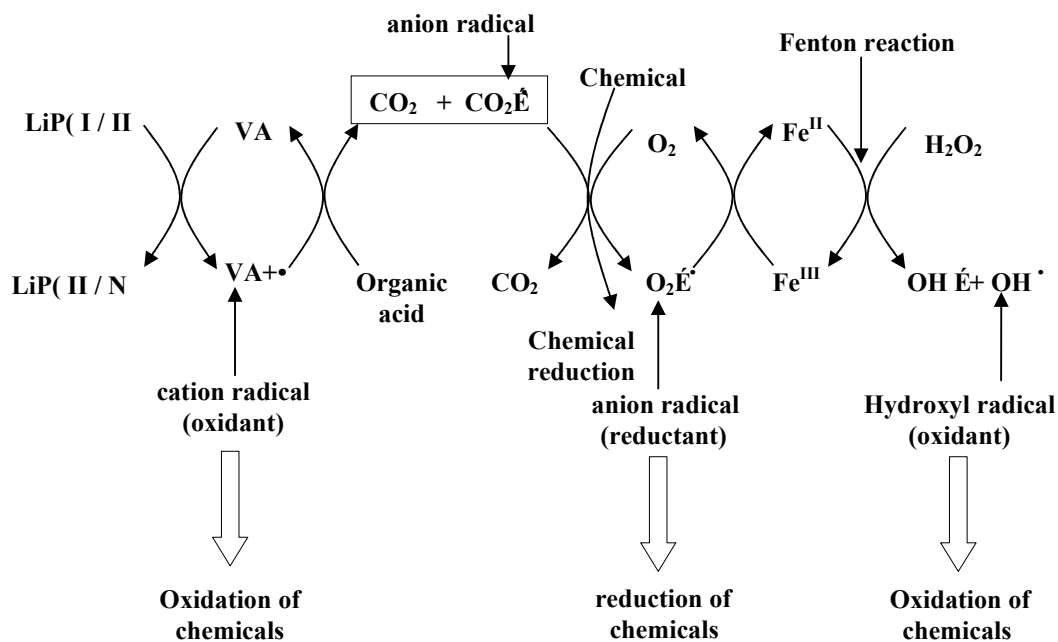


Figure 1b.3 Lignin peroxidase catalyzed reduction and generation of radical

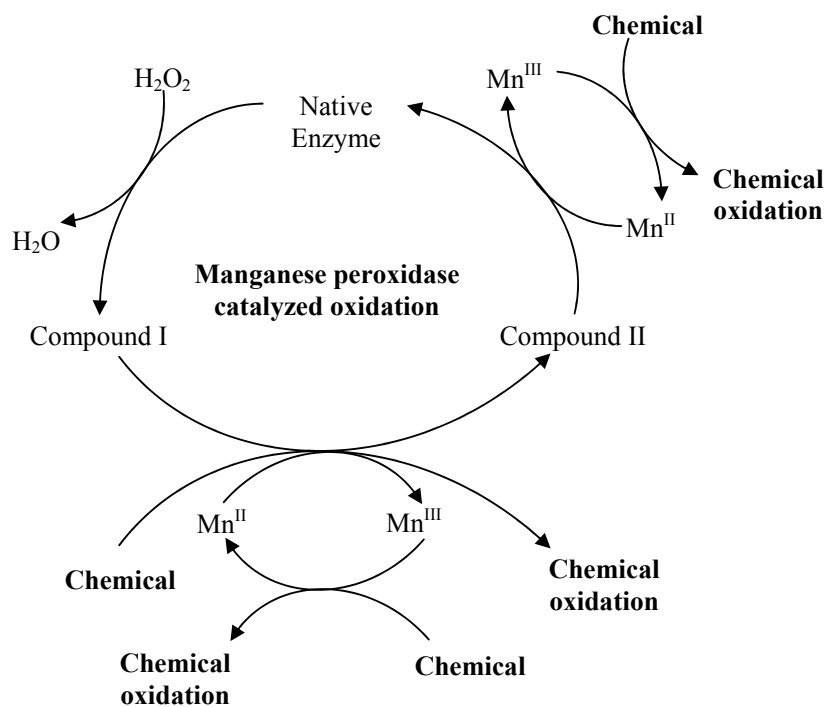


Figure 1b.4 Manganese peroxidase catalyzed oxidation

Chelates of Mn^{3+} with carboxylic acids (oxalate, malonate, malate, tartrate, lactate) cause one-electron oxidation of various substrates. Phenolic and amino-aromatic compounds are oxidized by hydrogen abstraction to form phenoxy and amino radicals, respectively (Glenn and Gold, 1985). Certain nonphenolic aromatic substances with low redox potential such as tetramethoxybenzene or anthracene are subject to one-electron abstraction from the aromatic ring, giving rise to aryl cation radicals (Sack *et al.*, 1997a). Chelates of Mn^{3+} and carboxylic acids can react with each other and are converted to alkyl radicals, which undergo subsequent spontaneous reactions with dioxygen resulting in the formation of other radicals (e.g. superoxide) (Hatakka, 2001). Versatile peroxidases produced by some WRF possess the ability to oxidize, in addition to Mn^{2+} also phenolic (phenol red) and nonphenolic (veratryl alcohol) aromatic compounds (Martinez *et al.*, 1996).

Reduction

MnP catalyzes reduction reactions in the presence of hydroquinones and Mn^{2+} (Chung *et al.*, 1993). Mn^{3+} oxidizes hydroquinones to corresponding semiquinone radicals, which has been shown to reduce more oxidized chemical. The quinone formed by this process is reduced back to hydroquinone by quinone reductases. Thus, highly oxidized pollutants, are indirectly reduced by MnP and LiP facilitating further metabolism (Chung *et al.*, 1993) (Fig. 1b.5).

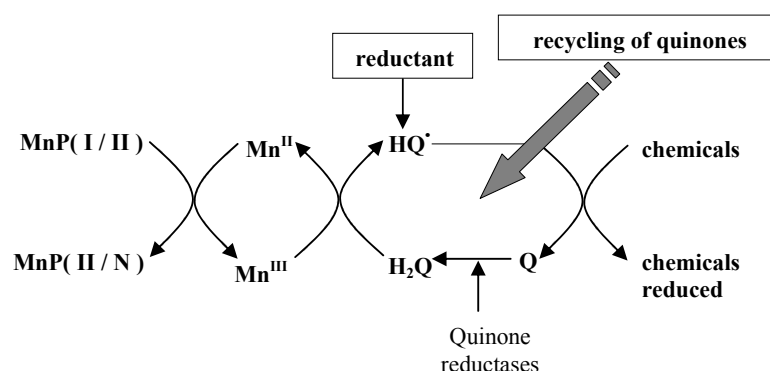


Figure 1b.5 Manganese peroxidase catalyzed reduction

(C) Methylation

White-rot fungi methylate a wide variety of phenolic compounds by a trans-membrane methyl transferase, which is reported to be a detoxification mechanism (Joshi and Gold, 1993). The primary methyl donors S-adenosylmethionine and methyl chloride, are

synthesized by these fungi (Harper and Hamilton, 1988). Upon methylation of hydroquinones resulting methylated products are oxidized such that the aromatic ring is opened; quinones being degraded instead to redox cycle (Valli *et al.*, 1992). Methylation of phenolic compound yields corresponding anisole (Barr and Aust, 1994). Phenols are not efficient substrates of LiP but *o*-methylated aromatic compounds such as veratryl alcohol are very efficiently oxidized by LiP. Chlorinated phenols such as 2,4,5-trichlorophenol and PCP are mineralized by WRF where methylation is the first step of such metabolism (Joshi and Gold, 1993). PCP, potent inhibitor of oxidative phosphorylation is quite toxic to multitude of organisms, upon methylation is converted to pentachloroanisole that is less toxic (Kirk *et al.*, 1992). Therefore, WRF use methylation as a mechanism to detoxify pollutants and following detoxification LiP and MnP effectively metabolize the pollutants further.

(D) Transmembrane redox potential

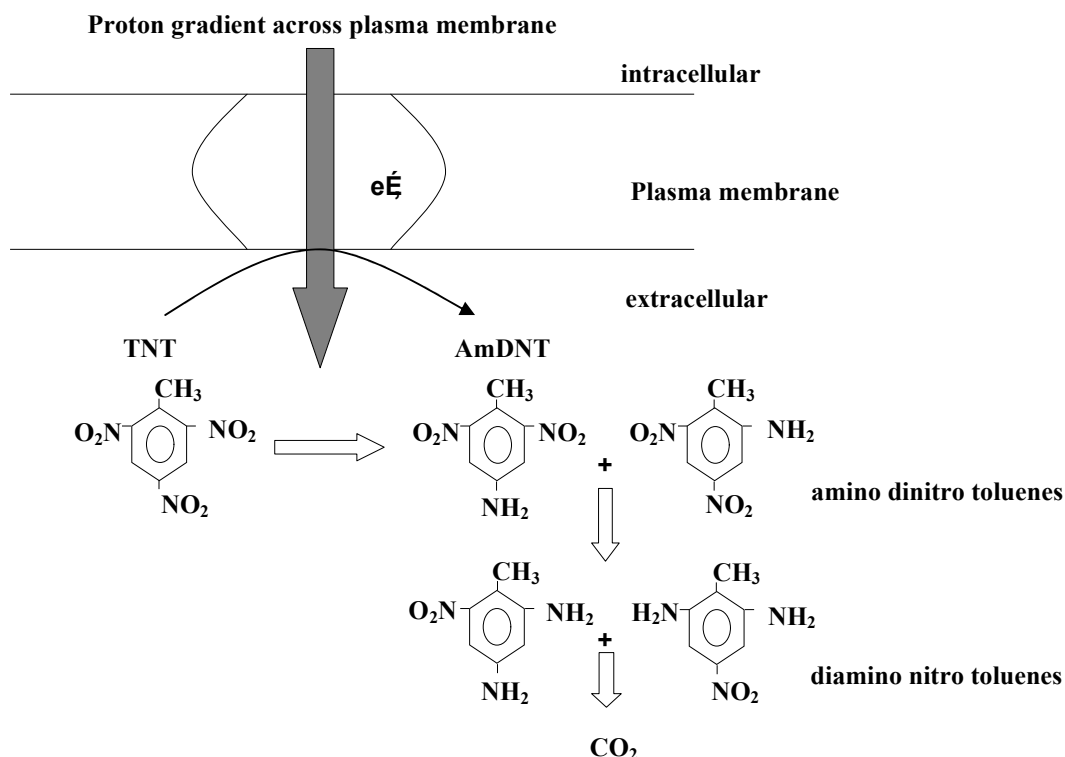


Figure 1b.6 TNT reduction by plasma membrane redox potential

A number of highly oxidized chemicals including TNT are reduced by cultures of WRF under non-ligninolytic conditions and the mechanism is independent of ligninolytic enzymes. A method of reduction used by many microbes, including filamentous fungi, involves maintenance of a proton gradient across the plasma membrane. TNT is reduced by cultures of *P. chrysosporium* to mono- and di-amino congeners by transmembrane redox potential (Fig. 1b.6), which are further oxidized by MnP and subsequent evolution of CO₂ is associated with the production of LiP (Stahl and Aust, 1993). The conversion of TNT to its amino congeners is a significant step in detoxifying TNT and its further metabolism.

XENOBIOTIC DEGRADATION

Polycyclic aromatic hydrocarbons (PAHs)

PAHs contaminate the environment via several routes, including improper disposal of wastes from the combustion of fossil fuels, coal gasification and liquefaction, incineration of industrial wastes, wood treatment processes and accidental spillage of petroleum hydrocarbons. PAHs have mutagenic, genotoxic and carcinogenic properties.

WRF degrade PAHs by non-specific oxidation, catalyzed by extracellular ligninolytic enzymes, that can further metabolize PAH quinones by cleaving the aromatic rings with subsequent breakdown of the PAH to CO₂ (Hammel, 1995). Since these enzymes are extracellular, have low substrate specificity and may diffuse into the soil matrix where the PAHs are entrapped, investigators have attempted to use ligninolytic fungi for the degradation of recalcitrant PAHs (Vyas *et al.*, 1994a; Novotny *et al.*, 1999).

The two main enzyme groups involved in the initial attack on PAHs by fungi are the cytochrome P-450 monooxygenase and LiP. Cytochrome P-450 incorporate one atom of molecular oxygen into the PAH molecule to form an arene oxide, which then undergoes either spontaneous isomerization to form a phenol, with subsequent conjugation with sulfate, glucuronic acid, glucose or xylase, or enzymatic hydration to form trans-hydrodiol (Cerniglia, 1993). LiP ionizes aromatic compounds to form aryl cation radicals, which undergo further oxidation to form quinones. Lignin peroxidase H8 from *Phanerochaete chrysosporium* is able to catalyze oxidation of 9-phenanthrol forming phenanthrene-9,10-quinone. 9-phenanthrol is an intermediate in the major pathway for

phenanthrene degradation under non-ligninolytic conditions, whereas the product, phenanthrene-9,10-quinone is an intermediate in degradation pathway under ligninolytic conditions (Tatarko *et al.* 1993). Purified forms of LiP and MnP have been shown to oxidize anthracene, pyrene, fluorene and benzo[a]pyrene to their corresponding quinones (Bogan, *et al.*, 1996; Hammel *et al.*, 1991). Some PAHs, up to six aromatic rings, are oxidized by manganese-dependent lipid peroxidation reactions, both *in vitro* and *in vivo* (Bogan *et al.*, 1996).

Trametes versicolor has been used in biodegradation studies because of its strong extracellular laccase production; the laccase of *T. versicolor* oxidises most of the 16 PAHs listed by the US EPA as priority pollutant chemicals. Benzo[a]pyrene and perylene are partially converted to polymeric products. Small amounts of quinones and ketones are the main oxidation products from anthracene, benzo[a]pyrene and fluorene (Collins *et al.*, 1996; Johannes *et al.*, 1996). The laccase of *T. versicolor* in combination with 1-hydroxybenzotriazole oxidizes acenaphthene and acenaphthylene to a variety of compounds; the primary metabolites are 1,2-acenaphthenedione and 1,8-naphthalic acid.

Bezalel *et al.*, (1996) demonstrated that *Pleurotus ostreatus* can degrade phenanthrene, anthracene, fluorene, pyrene and benzo[a]pyrene. The oxidation of anthracene by extracellular enzymes to 9,10-anthraquinone by different WRF has been demonstrated in several laboratories (Vyas *et al.*, 1994a). The other white-rot fungus *Bjerkandera* can oxidize a variety of PAHs including benzo[a]pyrene. A crude preparation of MnP from *Nematoloma frowardii* oxidized mixtures of eight PAHs, as well as five individual PAHs, including anthracene, phenanthrene, pyrene, fluoranthene and benzo[a]pyrene (Sack *et al.*, 1997a). Sack *et al.*, (1997b) screened several wood-decaying fungi, including *T. versicolor*, *Kuehneromyces mutabilis*, *Flammulina velutipes*, *Laetiporus sulphureus* and *Agrocybe aegerita* for phenanthrene and pyrene degradation. Many of the fungi screened were able to break down PAHs in liquid and straw cultures. Several other investigators have demonstrated the potential of white rot fungi for use in bioremediation of PAH contaminated soil (May *et al.* 1997, Bhatt *et al.* 2002).

Nitroaromatics and explosives

The three most widely used polynitroorganic compounds during the twentieth century are the highly energetic chemicals 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Presently soil and ground water contamination by explosives is a worldwide.

TNT, its metabolites, and related compounds exhibit considerable toxicity to humans, animals, and microorganisms. Only WRF producing the extracellular ligninolytic enzyme system are capable of oxidative destruction and mineralization of the aromatic nucleus of TNT. Ligninolytic fungi are able to mineralize TNT after they reduce it to aminonitro derivatives. Peroxidases are involved in the mineralization process, which is based on the enzymatic combustion of the aromatic nucleus. The magnitude of the enzymatic mineralization is in the order of 10 to 50%, depending on the experimental conditions. Recently, ninety-one fungal strains were tested and most were found capable of biodegrading TNT (Fritsche *et al.*, 2000). Several authors (Hofrichter *et al.*, 1999; Van Aken *et al.*, 1999) reported that MnP is capable of converting TNT and in particular its reduction products (hydroxylamino- and amino-dinitrotoluenes) to CO₂ in relatively high yields (4-80 %). The initial products from TNT biotransformation with *P. chrysosporium* were nitroso-toluene (NsT), *o*-hydroxylamino-4,6-dinitrotoluene (*o*-HADNT), *p*-hydroxylamino-2,4-dinitrotoluene (*p*-HADNT) and mono- and diaminotoluenes (ADNT and DANT) which were formed during the non-ligninolytic phase of the fungus. Several other secondary products were detected, including azo-, azoxy, phenolic, and acylated (acetylated and formylated) derivatives (Hawari *et al.*, 1999).

LiP or MnP generate reactive free radicals by an electron transfer process might enhance the degradation of the aromatic ring of TNT (Stahl and Aust, 1995; Fritsche *et al.*, 2000). Stahl and Aust, (1995) proposed the involvement of a membrane-bound redox system correlated with the proton secretion system in the reduction of TNT. Michels and Gottschalk, (1995) reported that the reductive activity is intracellular and depends on NADPH. Regardless of the site where TNT is reduced, further degradation and mineralization of TNT by *P. chrysosporium* occurs only when cultures are ligninolytic, implying that LiP, MnP and/or other enzymes of the ligninolytic system further transforms the reduced products of TNT (Hodgeon *et. al.*, 2000). The hydroxyaminodinitrotoluene products of TNT reduction inhibit the VAO activity of LiP.

The design of bioremediation process for TNT-contaminated soils with *P. chrysosporium* therefore requires conditions under which hydroxyaminodinitrotoluenes do not accumulate (Bumpus *et al.*, 1994).

The *in vitro* system is able to mineralize a mixture of reduction products from ^{14}C -U-ring-labeled TNT as well as ADNTs and 2,6-DANT. MnP catalyzes the oxidation of 2,6-DANT at a rate that is nearly tenfold higher than that of ADNTs. Van Aken *et al.*, (1997) published similar results for the oxidation of 4-HADNT, ADNTs, and DANTs by *Phlebia radiata*. Thus, a broad spectrum of reduction products of TNT serves as substrates of the MnP system. The transformation and mineralization of reduced derivatives of TNT by MnP are significantly enhanced in the presence of cysteine or reduced glutathione (GSH). It was proposed that a reactive complex of Mn(III)-GSH or Mn(II)-GS \cdot is the ultimate oxidant. MnP alone was always capable of affecting a low, but significant mineralization. It indicates that thiols and unsaturated fatty acids are the only enhancers of the MnP-catalyzed mineralization process (Hofrichter *et al.*, 1998).

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a military high explosive used alone, in a mixture with TNT or mixed with various plasticizers. Upto 12 mg/L could have been discharged to environment in process wastewater during the manufacture of RDX (Jackson *et al.*, 1978). Furthermore, the significant solubility of RDX in water and its limited adsorption to clay particles in soil makes its migration through soil to the water table a real concern. RDX exposure has been reported to cause epileptic seizures among exposed munitions workers (Testud *et al.*, 1996).

Biodegradation of RDX in liquid by *P. chrysosporium* approached 70% during the 24-day incubation, but mineralization slowed after 15 days. Degradation of RDX by MnP suggests oxidative degradation of the chemical. A hypothetical mechanism involving single electron oxidation of RDX by MnP generated Mn^{+3} destabilize the explosive sufficiently to undergo ring cleavage. RDX degradation by cellobiose dehydrogenase under anaerobic conditions suggested that WRF might also be able to employ a reductive mechanism similar to that observed with anaerobic organisms to degrade RDX (Stahl *et al.*, 2001). Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) occurs as the sole degradation product of RDX in the ligninolytic cultures of *P. chrysosporium*, (Sheremata and Hawari, 2000).

The mass-produced chemical warfare agent Yerpite [bis (2-chloroethyl) sulphide] has been shown to be completely mineralised by *T. versicolor*, although the role of the ligninolytic enzyme system is yet to be confirmed (Itoh *et al.*, 1997).

However, the ability of ligninolytic fungi to degrade a wide range of persistent xenobiotics, eg., TNT, RDX, HMX, provides a strong rationale for research and development of field applications. These fungi provide treatment systems that will operate *in situ* in large areas of contaminated soils (landfarming in combination with bioaugmentation and biostimulation by straw and wood chips). However, the potential toxicity of the products remains to be evaluated.

Halo-organics

Polychlorinated biphenyls (PCBs)

About 150 congeners have been reported in the environment. PCBs have entered into soil and sediment as a result of improper disposal of PCB wastes and leakage of PCB from electric transformers. PCBs persist in the environment and bioaccumulate because of their toxic, mutagenic, chemically inert and lipophilic nature. Therefore, their biodegradation is of environmental interest (Safe, 1994).

Removal of PCBs by incineration is expensive and brings additional risk of producing toxic chlorinated dioxins. *Phanerochaete chrysosporium* degrades chloroaromatic pollutants to CO₂ (Hammel, 1992) and low chlorinated congeners of PCB commercial mixtures Arochlor 1242, 1254 and 1260 (Sasek *et al.*, 1993; Vyas *et al.*, 1994b). Other WRF *B. adusta*, *Pl. osreatus* and *T. versicolor* exhibited higher biodegradation activity than *P. chrysosporium*. However, a direct correlation between the biodegradative ability and activities of ligninolytic enzymes is not yet conformed (Beaudette *et al.*, 1998). Yadav *et al.*, (1995) described PCB degrading ability of *P. chrysosporium* under both ligninolytic and non-ligninolytic conditions. Kremar and Ulrich, (1998) observed degradation of a PCB technical mixture under non-ligninolytic conditions and no degradation under ligninolytic conditions. In some studies, a high level of PCB removal was correlated with high ligninolytic enzymes production by fungi (Novotny *et al.*, 1997). Kremar *et al.*, (1999) demonstrated extracellular nature of PCB-degrading agent eliminating the role of MnP or LiP.

The ability of WRF to mineralise PCBs to CO₂ was tested using [¹⁴C] labelled PCBs (Vyas *et al.*, 1994b). In liquid media, up to 11% of the amount of PCB 31 was mineralised by *T. versicolor* (Beandette *et al.*, 1998). In soil, only 0.4% and less than 0.1% of PCB 11 and PCB 77 were mineralised by *Pl. ostreatus*, respectively (Kubatova *et al.*, 1998). Different extent of PCB transformation and mineralization is perhaps because of different specificities of enzymes and or different mechanisms employed for the degradation (Beandette *et al.*, 1998; Kubatova *et al.*, 2001).

The dehalogenation of PCBs by a commercial preparation of HRP and purified laccase of *T. versicolor* has been demonstrated (Dec & Bollag, 1995) and this is probably due to ligninolytic system mediated free radical production. Future research needs to be concentrated on PCB degradation mechanism and reaction products which remain unknown.

Chlorophenols

Chlorophenols are toxic, in some cases, highly persistent priority pollutants of aqueous systems, soils and solid waste material. Furthermore, they are major contaminants in effluents of pulp and paper industries (Valli and Gold, 1991). Ligninolytic enzymes namely laccase, LiP and MnP carry out initial oxidation of chlorophenols (Valli and Gold, 1991). LiP and MnP have been observed to catalyse subsequent oxidation of metabolites formed during chlorophenol degradation, such as hydroquinones and methylated derivatives (Ricotta *et al.*, 1996). The oxidation of chlorophenols by such enzymes results in the formation of phenoxy radicals, followed by spontaneous subsequent reactions leading to the formation of quinones and several di- and oligomeric coupling products. (Valli and Gold, 1991). These processes are accomplished by dechlorination to a certain extent, depending on the respective chlorophenol (Dec & Bollag, 1994).

MnP has been found capable of directly but partially mineralising 2,4,-dichlorophenol (2,4-DCP) in the cell free system. Higher degree of chlorination is preferred by MnP. (Hofrichter *et al.*, 1998). 2,4-DCP is most efficiently oxidised by combination of MnP and laccase. Oxidation by MnP and laccase alone is less effective (Schlosser *et al.*, 2000). 2,4-DCP is oxidised by LiP (Valli and Gold, 1991). It has also been shown that *P. chrysosporium* most efficiently mineralises several chloro-, methyl- and ethyl- substituted

benzene under non-ligninolytic conditions where LiP and MnP are not expressed (Yadav *et al.*, 1995).

4,5-dichlorocatechol appears during the degradation of 3,4-DCP by *P. chrysosporium* under non-ligninolytic condition, is attributed to an intracellular hydroxylase (Deschler *et al.*, 1998). Cell-bound reductive dechlorination of chlorinated and hydroxylated benzene is also reported in this fungus (Reddy *et al.*, 1998). 2,4-DCP and 2,4,6-trichlorophenol efficiently mineralized under conditions in which LiP and MnP are expressed (Valli and Gold, 1991; Joshi and Gold, 1993). Reddy *et al.*, (1998) discovered that reductive dechlorination reactions are involved in the degradation of 2,4,6-trichlorophenol by *P. chrysosporium* and several other white rot fungi. (Schlosser *et al.*, 2000).

Pesticides

Pentachlorophenols (PCP)

PCP, a broad spectrum biocide, is used as a fungicide, insecticide, herbicide, algicide, disinfectant and antifouling agent (Crosby, 1981). The use of pesticides and other chemicals give rise to residues in surface and groundwater, sewage and soils.

Cell free activity observed in the *P. chrysosporium* cultures identified as LiP, catalyzed conversion of PCP to 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (Mileski *et al.*, 1988). Soil cultures of *P. chrysosporium* have been found to *o*-methylate PCP (Lamer *et al.*, 1990). LiP or MnP oxidatively dechlorinate PCP to tetrachloro-1,4-benzoquinone. The quinone is further reduced to tetrachlorodihydroxybenzene, which can undergo four successive dechlorinations to produce 1,4-hydroquinones and further hydroxylated to form 1,2,4-trihydroxybenzene. Alternatively, tetrachloro-1,4-benzoquinone is converted either enzymatically or non-enzymatically to 2,3,5-trichlorohydroxybenzene, which undergoes successive reductive dechlorinations to produce 1,2,4-trihydroxybenzene. The final product produced in each pathway is ring cleaved with subsequent degradation to CO₂ (Reddy and Gold, 2000). Key intermediate tetrachlorohydroxybenzene is readily degraded under both nitrogen-sufficient and nitrogen-limiting conditions, suggesting that other enzymes are involved in PCP degradation pathways and extracellular peroxidases apparently are involved only in the initial step, the oxidation of PCP to tetrachlorobenzoquinone.

DDT

DDT [1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane] was among the first persistent organopollutants demonstrated to be degraded by *P. chrysosporium*. Nitrogen limited cultures of *P. chrysosporium* mineralise ^{14}C -DDT completely resulting in the formation of several metabolites and evolution of $^{14}\text{CO}_2$. *Pl. ostreatus*, *Phellinus weirii* and *Polyporus versicolor* are also capable of mineralising DDT. The intermediates identified as DDT metabolites were DDD, FW-152, dicofol and 4,4'-dichlorobenzophenone (Bumpus & Aust, 1987).

Biodegradation of DDT can result in toxic and persistent metabolites. One study has shown that ^{14}C -radiolabelled 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene (DDE), extremely toxic and persistent DDT breakdown product is mineralized to $^{14}\text{CO}_2$ by *P. chrysosporium* (Bumpus *et al.*, 1993a). 2,7-dichlorodibenzo-*p*-dioxin is also mineralized by *P. chrysosporium* (Valli *et al.*, 1992). Purified LiP and MnP in a multi-step pathway involving sequential oxidation, reduction and methylation reactions remove the two Cl atoms and carry out ring cleavage and mineralization. Biodegradation of the organochlorine herbicide 2,4,5-T and 2,4-D by *P. chrysosporium* has been demonstrated (Ryan and Bumpus, 1989; Yadav and Reddy, 1993), although the role of ligninolytic enzymes in this process was not confirmed.

The chlorinated triazine herbicide 2-chloro-4-ethylamine-6-isopropyl-amino-1,3,4-triazine (atrazine) is transformed by *P. chrysosporium* and *Pl. ostreatus* to hydroxylated and N-dealkylated metabolites (Mougin *et al.*, 1994; Masaphy *et al.*, 1993). *P. chrysosporium* has been demonstrated to mineralise ^{14}C -radiolabelled organophosphate insecticides chlorpyrifos, fonofos and terbufos during 18-day incubation without the involvement of ligninolytic enzymes (Bumpus *et al.*, 1993b).

Synthetic Dyes

Synthetic dyes are extensively used in textile, paper, photography, cosmetic and leather industries. Over 100,000 different dyes and pigments exist and over 7×10^5 tons of dyestuffs are annually produced worldwide (Zollinger, 1987). Between 2-50% of the total dye consumed in dyeing process may be found in wastewaters depending on the class of dye application (O'Neill *et al.*, 1999). These dyes include several structural varieties such as acidic, reactive, basic, disperse, azo, diazo, anthraquinone-based and metal-complex

dyes. Effluents discharged from textile and dyestuff industries to neighbouring water bodies are causing significant health concerns (Banat *et al.*, 1996). Interest in pollution potential of synthetic dyes has primarily been prompted by concern over their possible toxicity and carcinogenicity.

The decolorization of dyes by WRF was first reported by Glenn and Gold (1983). *P. chrysosporium* decolorizes polymeric dyes, azo and heterocyclic dyes (Cripps *et al.*, 1990; Ollikka *et al.*, 1993). Involvement of lignin-degrading enzymes in the decolorization of dyes was investigated by several workers (Paszczyński & Crawford, 1991; Ollikka *et al.*, 1993). Podgornik *et al.*, (1995) studied decolorization of 50 structurally different dyes by extracellular LiP of *P. chrysosporium*. They showed that decolorization could proceed by either a uniform decrease in the absorbance through the spectrum or formation of a new transient absorption peak. Differential expression of LiP isoforms by different taxa or culture conditions may result in variable dye-decolorizing ability.

Decolorization of dyes by ligninolytic enzymes is an oxidative process that can result in complete degradation of the dye molecule to CO₂ and H₂O. (Spadaro *et al.*, 1992). The oxidative decolorization of methylene blue by LiP from *P. chrysosporium* was demonstrated by Kling and Neto (1991). It was originally assumed that MnP and laccase would only convert a limited spectrum of azo dyes and preferentially convert dyes which carry a phenolic substituent in para-position to the azo bond and additionally methyl- or methoxy- substituents in 2- or 2,6- position in relation to hydroxy- group (Chivukula & Renganathan, 1995). Later it was shown that MnPs of *B. adusta* and *Pl. eryngii* catalyse dye decolorization. The enzymes from both fungi were unusual in that they did so in Mn²⁺-independent reaction (Heinfling *et al.*, 1998). Recently Moreira *et al.* (2001) demonstrated decolorization of Poly R-478 using MnP. Laccase from *Pycnoporus cinnabarinus* is able to decolorize complex, industrially relevant azo dyes such as Reactive Blue 5 and Direct Blue 1 (Schliephake *et al.*, 2000).

Recently, Jarosz-Wilkolazka *et al.*, (2002) screened 115 fungi of different physiological group for their ability to decolorize azo and heterocyclic dyes, concluding that anthraquinone dyes are decolorized easier and faster by fungi than azo dyes. Laccases from *T. versicolor* and *Polyporus pinisitus* were found to decolorize several dyes efficiently both in the presence and absence of redox-mediator 1-hydroxybenzotriazole,

which improved & facilitated the decolorization (Claus *et al.*, 2002). A bioreactor packed with *P. chrysosporium* immobilized on polymethane foam presented good stability with high decolorization percentages of a hardly biodegradable dye Poly R-478 (Mielgo *et al.*, 2002). Recently Selvam *et al.* (2003) reported the decolorization of azo dyes and dye industry effluents by *Thelephora* spp. Our group recently reported the decolorization of sulfonphthalein dyes by ligninolytic enzymes of different WRF (Christian *et al.*, 2003; Shrivastava *et al.*, 2004). Involvement of degradative mechanism other than ligninolytic enzyme for dye decolorization has also been studied. Pasti & Crawford (1991) proposed plasma membrane redox system of WRF for dye decolorization. Kirby (1999) evidenced that such a mechanism was involved in decolorization of Remazol Black B by strains of *P. chrysosporium* and *T. versicolor*. Vyas and Molitoris (1995) reported decolorization of RBBR by a novel enzyme produced by *P. ostreatus*, which is different from MnP, LiP, VAO and laccase. The activity was named RBBR oxygenase. They reported that dye decolorization by WRF undergoes sequential change of blue dye to colorless through a rainbow of intermediates also shown by other researchers (Kirby, 1999). It is also proposed from such observations that decolorization is not a single step reaction and intermediates are involved during complete decolorization.

Due to the inherent complexity of both the dye molecules and enzymatic machinery involved, the degradative pathways utilized by WRF remain mostly unelucidated. Conneely *et al.*, (1999) attempted to elucidate the degradation pathway of copper-phthalocyanine dyes by *P. chrysosporium*. Dye structure was readily degraded and both free copper and organo copper breakdown products were found in culture supernatants. Spadaro and Renganathan (1994) reported that the oxidation of non-sulfonated azo dyes [1-(4'-acetamidophenylazo)-2-naphthol] by LiP from *P. chrysosporium* resulted in the formation of 1,2-naphthaquinone and acetanilide. Two groups reported degradation of different sulfonated azo dyes by crude and purified peroxidase preparations, respectively. (Goszczynski *et al.*, 1994; Chivakula and Renganathan, 1995).

Decolorization of polymeric dyes has been proposed as a useful screening method for ligninolytic activity. Field *et al.*, (1993) revealed good correlation between PAH degradation and Poly R-478 decolorization rates. RBBR and polymeric dyes are therefore being used for measuring ligninolytic activities (Sasek *et al.*, 1998; Novotny *et al.*, 2001). Degradation of such dyes correlated with the initiation of lignin metabolism and reflects a

combined effect of peroxidases and H₂O₂-producing oxidases (Glenn and Gold, 1983). Therefore, the dye decolorization studies are being used as a possible, easily usable and inexpensive alternative to radiolabelled lignins and other xenobiotics in biodegradation studies.

Summary

The mechanisms described here make the WRF technology unique among more established methods of bioremediation. The differences are primarily due to mechanisms discussed previously. The unusual mechanisms used by the fungi provide them with several advantages for pollutant degradation. The ability of WRF to degrade structurally diverse xenobiotic organopollutants is demonstrated in number of experiments both in liquid media and under soil conditions. Additionally various species of fungi that produce MnP, LiP, laccase and other enzymes may permit the use of numerous indigenous or introduced fungi for remediation purpose. Despite valuable basic knowledge on the mechanism of pollutant biodegradation, bioremediation has not yet been accepted as a routine treatment technology and environmental industry is wary in applying bioremediation. Thus, both more extensive and intense research especially for searching and exploiting new fungal species and improvement of practical application is needed to establish mycoremediation as an effective and reliable bioremediation technology.

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CHAPTER 2

DECOLORIZATION OF TEXTILE DYES AND DYE INDUSTRY EFFLUENTS BY *Irpex lateus*

2.1 Introduction

Dyes constitute an important family of organopollutants since they find a wide variety of uses in everyday life and represent structurally and chemically diverse classes of xenobiotic compounds. Dyes are usually aromatic and heterocyclic compounds and are often recalcitrant. Some of them are toxic and even carcinogenic. Large amounts of dyes are used for various industrial applications including dyeing and printing. During textile processing, inefficiencies in dyeing result in large amount of the dyestuff being directly lost to the wastewater and that gets spilled into the environment (Meyer, 1981). Conventional wastewater treatment is not efficient to remove recalcitrant dyestuffs from effluents (Shaul *et al.*, 1991). Biodegradation is an environment-friendly and cost competitive alternative.

In recent years the possibility of using white rot fungi for bioremediation strategies has initiated considerable research efforts. The interest in this subject arises from the ability of white rot fungi to degrade most recalcitrant natural polymer lignin and an extremely diverse range of highly persistent or toxic environmental pollutants. This ability sets the use of white rot fungi apart from many of the existing methods of bioremediation.

Eaton *et al.*, (1980) reported for the first time biological decolorization of wastewaters from pulp and paper industries using *Phanerochaete chrysosporium* and *Tinctoporia* spp. Since then much of the dye decolorization work has been concentrated on *P. chrysosporium*, which has been reported to decolorize triphenylmethane, azo, heterocyclic and polymeric dyes (Ollikka *et al.*, 1993). Fungal ligninolytic enzymes lignin peroxidase (LiP), manganese peroxidase (MnP), manganese-independent peroxidase and laccase, have been repeatedly implicated in

decolorization of diverse synthetic dyes (Ollikka *et al.*, 1993; Vyas and Molitoris, 1995; Christian *et al.*, 2003; 2004).

There are very limited reports available on decolorization of effluents by white rot fungi (Zhang *et al.*, 1998; Selvam *et al.*, 2003). In preliminary studies, the decolorization of textile dyes was evaluated using three different fungi. *Irpex lacteus* was the focus of this study, as it was found to be unique in its ability to decolorize all textile dyes. *Irpex lacteus* is a cosmopolitan white rot fungus, and was used to study its potential to decolorize textile dyes as well as complex effluents from textile dye industries.

2.2 Materials and methods

Microorganisms

Three ligninolytic fungi *Irpex lacteus* Fr. 238 617/93, *Agrocybe cylindracea* 081 and *Pleurotus ostreatus vampola* originated from Culture Collection of Basidiomycetes (CCBAS), Prague were used (Kindly gifted by Ša ek, V.). The strains were maintained on malt extract agar plates at 5 °C.

Textile dyes and effluents

Seven different textile dyes Remazol magenta HB (A₅₅₇), Reactive blue 21 (A₆₂₀), Remazol red H8B (A₅₀₉), Reactive orange 13 (A₄₈₀), Reactive brown 18 (A₄₅₂), Reactive black 5 (A₅₉₆) and Remazol brilliant blue R (A₅₉₅) were used for decolorization studies. Effluents designated as E-1, E-2, E-3 collected from three dyeing industries using above dyes were also used.

Decolorization of textile dyes on solid media

The three fungi were screened for their ability to decolorize textile dyes using malt extract agar medium (malt extract 30 g l⁻¹, mycological peptone 5 g l⁻¹ and agar 15 g l⁻¹) containing the dyes (100 ppm). The plates were inoculated with a disk (8 mm) punched from malt extract agar plate cultures (7 day) of the three fungi. Relative growth rate was calculated as a ratio of zone of growth on malt agar media without and with dye.

Inoculum

Ten disks (8 mm) cut from cultures (7 day) growing on malt extract agar plates were used to inoculate 100 ml modified Kirk's medium containing Tween 20 (0.5 %) in 250 ml Erlenmeyer flasks. Peptone (0.5 g.l⁻¹) and yeast extract (0.1 g.l⁻¹) were added instead of ammonium tartarate and acetate buffer was used instead of dimethyl succinate buffer. The flasks were incubated on an orbital shaker (28 °C, 100 rpm) for 14 days. The contents of the flasks were gently homogenized and 5 ml was used to inoculate 100 ml medium with dye or effluent in each flask.

Decolorization of textile dyes and effluents in liquid media

The decolorization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml modified Kirk's medium containing respective dyes (100 ppm). Medium for effluent decolorization was prepared by adding medium components directly to effluents except for E-2 where effluent was diluted 1:2 with distilled water and pH was adjusted to 4.5. The inoculated flasks were incubated on an orbital shaker (28 °C, 100 rpm). Appropriate biotic and abiotic controls were also included in the experiments.

Samples (3 ml) were withdrawn at designated time intervals and centrifuged (5000 rpm, 15 min, 4 °C). The clear supernatant was analyzed for pH, residual dye and ligninolytic enzyme activities. Decolorization was estimated by measuring the decrease in absorbance at the λ_{max} of respective dyes and spectra (400–800 nm) were recorded using Shimadzu UV-1601 spectrophotometer.

Enzyme assays

Manganese peroxidase (MnP), manganese-independent peroxidase (MIP), and laccase activities were determined as described earlier (Vyas et al., 1994). Lignin peroxidase activity was estimated according to Tien and Kirk (1984). One unit of enzyme activity is defined as the amount of activity that will produce one μmol of the product per min upon oxidation of the substrate in the reaction mixture under assay conditions.

2.3 Results and discussion

Screening on solid media

Irpex lacteus, *Agrocybe cylindracea* and *Pleurotus ostreatus vampola* were screened for their ability to decolorize textile dyes on malt agar medium. Inhibition of fungal growth by textile dyes was also evaluated by comparing the time required to colonize the whole plate with and without dye. The relative growth rates for all dyes were calculated (Table 2.1).

Dyes	Relative growth rate to colonize the whole plate ^a		
	<i>Irpex lacteus</i>	<i>Agrocybe cylindracea</i>	<i>Pleurotus ostreatus vampola</i>
Remazol magenta HB	1	1	1.3
Reactive blue21	1.3	1.8	2.7
Remazol red H8B	1	1.6	1.9
Reactive orange 13	1.8	1.2	2.1
Reactive brown 18	1.3	1.6	1.6
Reactive black 5	1	1	1
Remazol brilliant blue R	1	1	1

^a Relative growth rate is a ratio of days required to colonize the whole plate without dye to that of with day.

Table 2.1 Inhibitory effects of dyes on growth of ligninolytic fungi on agar media

Relative growth rate of *I. lacteus* on media containing reactive blue 21, reactive orange 13 and reactive brown 18 was marginally higher than 1 implying that these dyes were slightly inhibitory (Table 2.1). Initiation of decolorization and completion of decolorization was first observed with remazol magenta HB and reactive black 5 by *I. lacteus*. Decolorization of remazol red H8B and remazol brilliant blue R started as early as day 4 and completed by day 8. *Irpex lacteus* decolorized reactive orange 13 and reactive brown 18 by day 11 and shed of reactive blue 21 remained even after 20 days. Decolorization of dyes and relative growth rate of *A. cylindracea* was almost similar as *I. lacteus* (Table 2.2).

Dyes	Decolorizing activity ^a		
	<i>Irpex lacteus</i>	<i>Agrocybe cylindracea</i>	<i>Pleurotus ostreatus vampola</i>
Remazol magenta HB	D (6)	D (6)	D (12)
Reactive blue21	++ (20)	++ (20)	- (20)
Remazol red H8B	D (8)	D (18)	++ (20)
Reactive orange 13	D (11)	D (11)	++ (20)
Reactive brown 18	D (11)	D (18)	D (18)
Reactive black 5	D (6)	D (7)	++ (20)
Remazol brilliant blue R	D (8)	D (7)	D (10)

^a Decolorizing activity: (-) no decolorization, (+) partial decolorization, (++) almost complete decolorization, (D) completed decolorization without any dye shade. Numbers in parentheses indicate the day of incubation on which maximal degree of decolorization was accomplished.

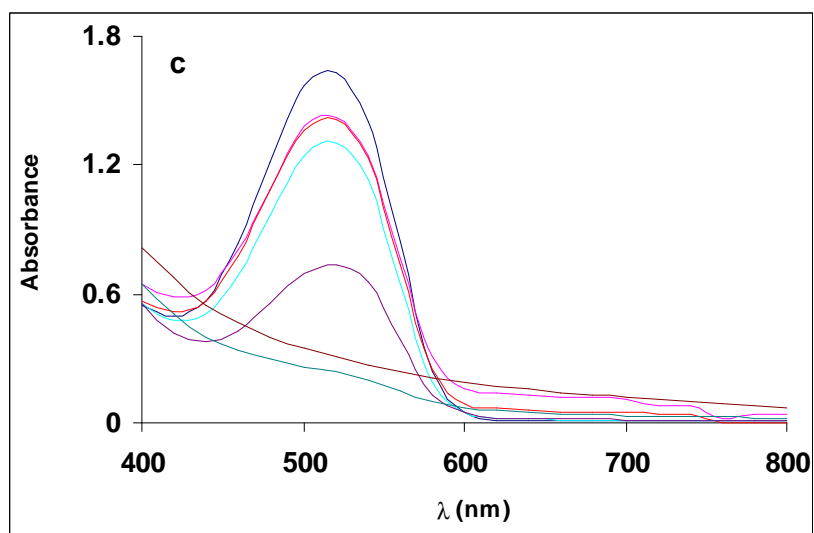
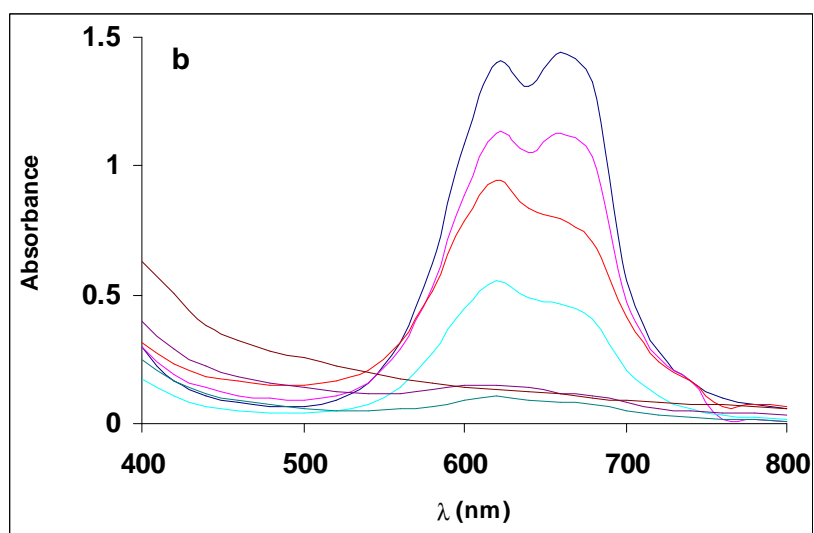
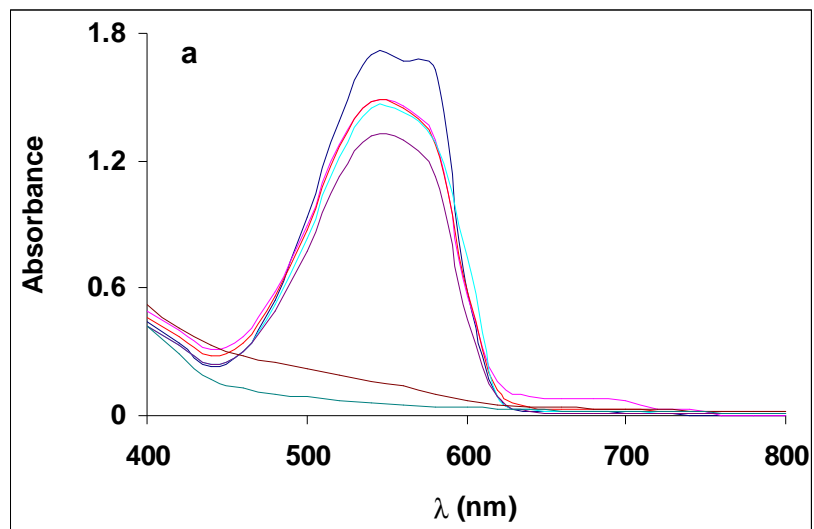
Table 2.2 Decolorization of textile dyes by ligninolytic fungi on agar media

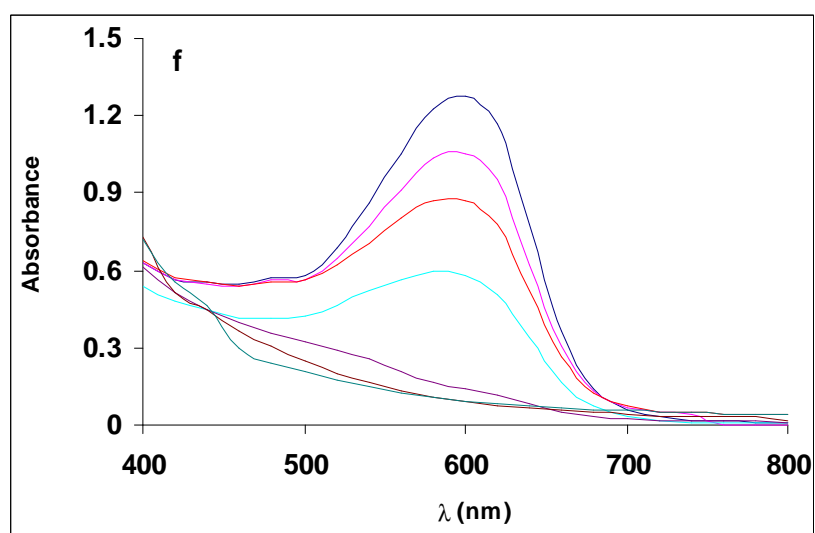
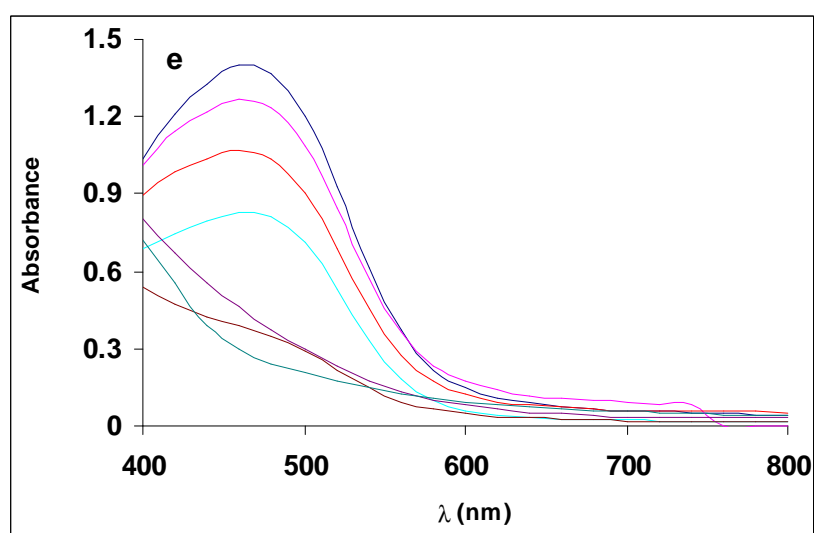
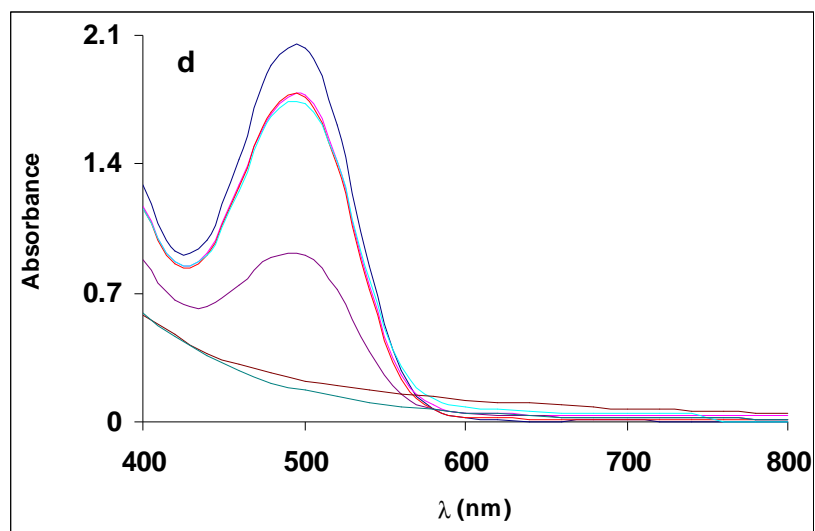
Relative growth rate of *P. ostreatus* was higher with reactive blue 21, reactive orange 13 and remazol red H8B, indicating inhibition of growth by these dyes (Table 2.1). *Pleurotus ostreatus* decolorized remazol red H8B, reactive orange 13 and reactive black 5 almost completely but did not decolorize reactive blue 21. Remazol magenta HB and remazol brilliant blue R were completely decolorized by *P. ostreatus* (Table 2.2).

Irpex lacteus that efficiently decolorized all textile dyes was selected for further study.

Decolorization of textile dyes

Decolorization was measured as decrease in absorbance at the λ_{\max} of respective dyes. Visible spectra were measured of all dyes during the experimental run (Fig. 2.1). The decrease in absorbance was a function of time during the growth of *I. lacteus* on medium containing dyes and effluents.





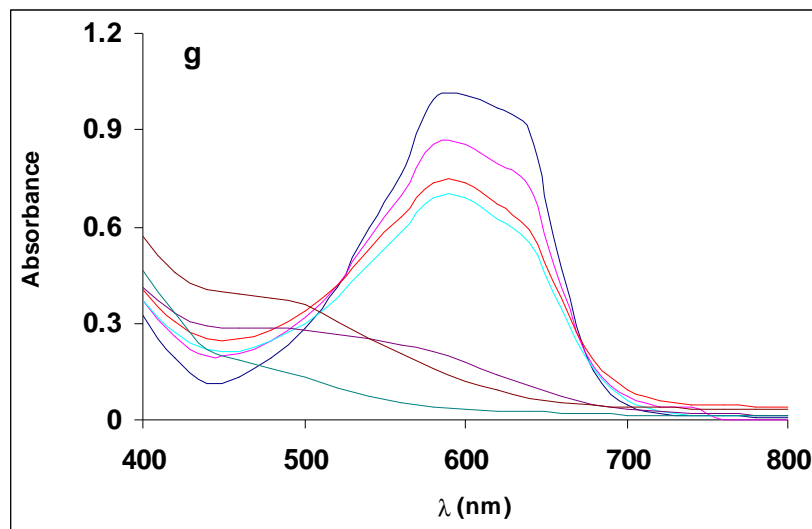


Figure 2.1 Visible spectra of dyes (a) Remazol magenta HB; (b) Reactive blue 21; (c) Remazol red H8B; (d) Reactive orange 13; (e), Reactive brown 18; (f) Reactive black 5; (g) Remazol brilliant blue R by submerged cultures of *Irpex lacteus*.: Control (—); Day 3 (—); Day 6 (—); Day 9 (—); Day 12 (—); Day 15 (—); Day 18 (—).

Knapp et al., (1995) reported that the dyes that are structurally similar may be differentially decolorized by white rot fungi. The reason was postulated to be due to electron distribution and charge density along with the steric distribution. Remazol magenta HB was only partially decolorized upto day 12 and extensively decolorized by day 18 leading to complete loss of the color in the medium. *Irpex lacteus* decolorized reactive blue 21 gradually along the experimental run and was completely decolorized in contrast to its decolorization on solid media. More than 50% of remazol red H8B and reactive orange 13 were decolorized by Day 12. Decolorization of reactive brown 16 was comparatively slow. Decolorization of remazol magenta HB, remazol red H8B, reactive orange 12 and remazol brilliant blue R was slow till day 9 when rest of the dyes were decolorized by 50%. Reactive blue 21, reactive black 5 and remazol brilliant blue R were completely decolorized by day 12 (Fig. 2.1).

Upon decolorization absorbance maxima of all the dyes tested shifted towards lower wavelength indicating hypsochromic shift. This implies that the decolorization was not due to the adsorption of the dyes to fungal mycelium but was the outcome of degradation of the dye through a series of intermediates (Gold *et al.*, 1988; Vyas and Molitoris, 1995).

Decolorization of textile industry effluents

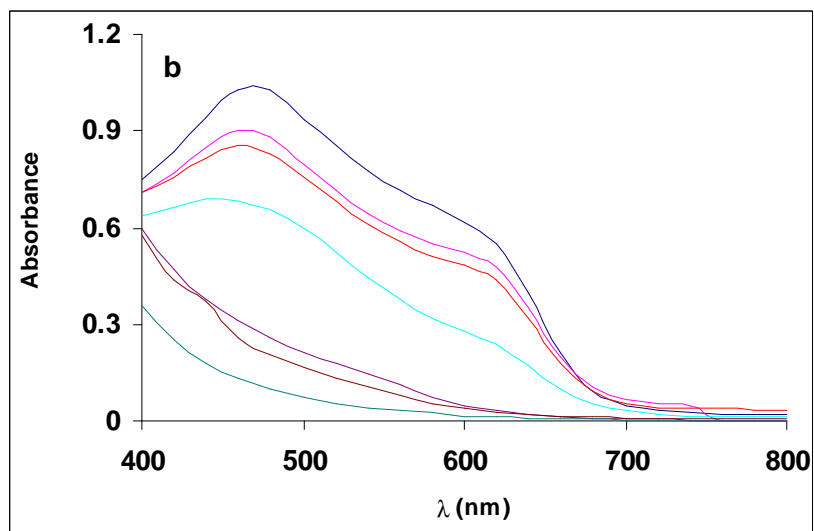
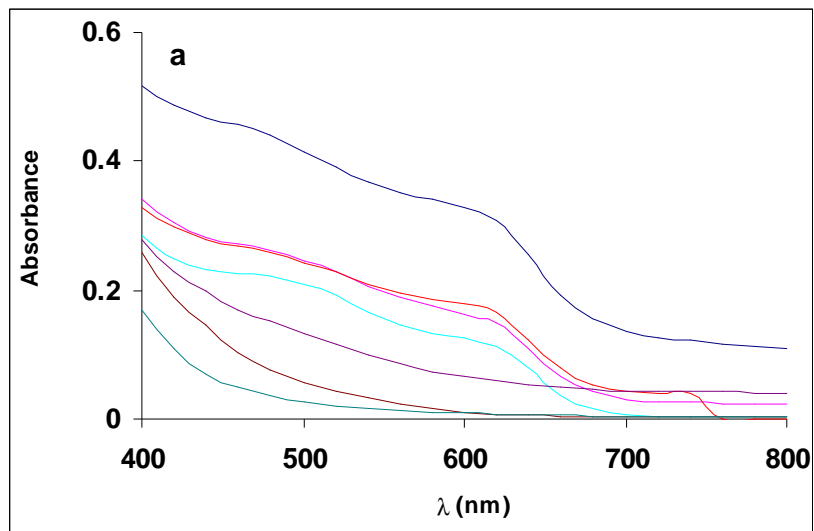
The physical and chemical parameters of the effluents collected from three different textile industries (E-1, E-2 and E-3) were characterized according to the standard methods (Eaton *et al.*, 1995) (Table 2.3). These effluents also contained the binding agent silicate and other chemicals used in the dyeing industries.

Physico-chemical parameter	E-1	E-2	E-3
pH	6.45	6.32	6.8
Conductivity	5.79	4.72	5.24
Turbidity (NTU) ^a	0.4	2.4	1.3
Total dissolved solids	440	700	360
Copper (ppm)	0.022	0.023	0.081
Nitrite (ppm)	26	29	38
Zinc (ppm)	2.9	2.1	3.9
Chloride (ppm)	38	69	86
Sulfate (ppm)	666.4	799	780.9

Table 2.3 Physicochemical characterization of effluents from textile dye industries.

Submerged cultures of *I. lacteus* decolorized effluents E-1, E-2 and E-3 extensively within 18 days. Decolorization proceeded by a uniform decrease in the absorbance throughout the visible spectrum with all the effluents (Fig. 2.2). Recently Selvam *et al.*, (2003) reported decolorization of azo dye industry effluents in liquid medium and by pure enzymes. Higher degree of decolorization was achieved with the whole cultures than ligninolytic activities (purified or

partially purified) suggest that whole cultures are more effective because of the involvement of all the enzymes along with non-enzymatic components of ligninolytic system.



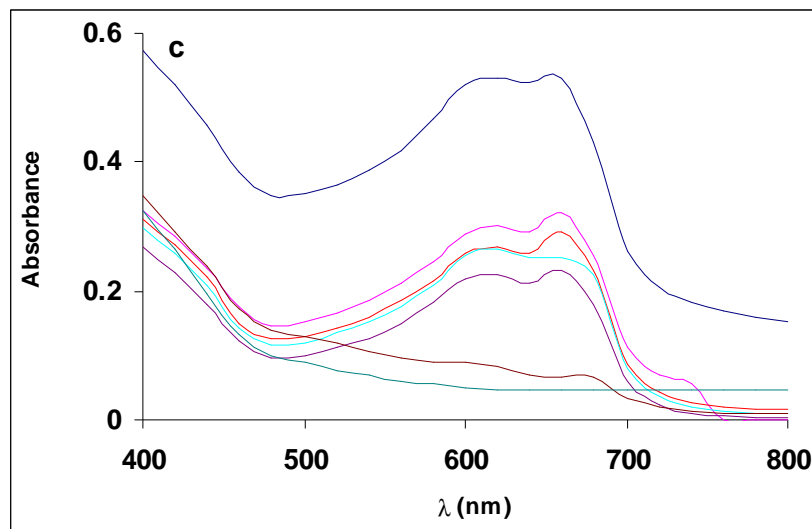


Figure 2.2 Visible spectra of dyes (a) Effluent-1; (b) Effluent-2; (c) Effluent-3 by submerged cultures of *Irpex lacteus*.: Control (—); Day 3 (—); Day 6 (—); Day 9 (—); Day 12 (—); Day 15 (—); Day 18 (—).

Ligninolytic enzyme production

Many researchers have reported involvement of ligninolytic enzymes in the decolorization of several dyes (Ollikka *et al.*, 1993; Christian *et al.*, 2003; Vyas and Molitoris, 1995). We analyzed the correlation between the production of ligninolytic activities and dye decolorization. *Irpex lacteus* produced manganese peroxidase (MnP) (Fig. 2.3), manganese-independent peroxidase (MIP) (Fig. 2.4), lignin peroxidase (LiP) (Fig. 2.5) and laccase (Fig. 2.6) as early as day 3.

High amount of MnP was accumulated in the medium during day 15-18. MnP activity in the cultures with reactive blue 21, remazol red H8B, reactive brown 18 and remazol brilliant blue R was as high as control. The trend of MIP and laccase was similar to that of MnP. It was interesting to observe that LiP activity was detectable as early as day 3 but in the cultures containing reactive orange 13, reactive brown 18 and remazol brilliant blue R it appeared on day 6. All ligninolytic activities produced in control and in the cultures with dyes were comparable.

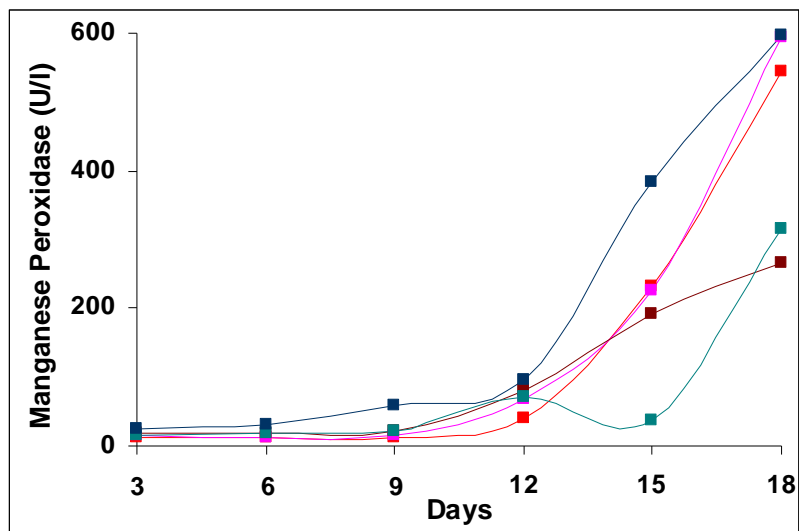


Figure 2.3 Manganese peroxidase production by *Irpex lacteus* in the absence and presence of dye: Without dye (—); Remazol brilliant blue R (—); Reactive brown 18 (—); Effluent-1 (—); Effluent 3 (—).

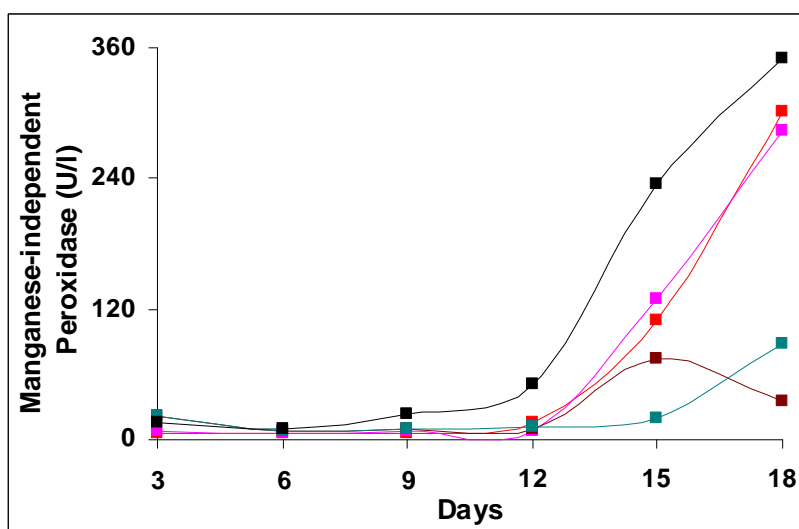


Figure 2.4 Manganese-independent peroxidase production by *Irpex lacteus* in the absence and presence of dye: Without dye (—); Remazol brilliant blue R (—); Reactive brown 18 (—); Effluent-1 (—); Effluent 3 (—).

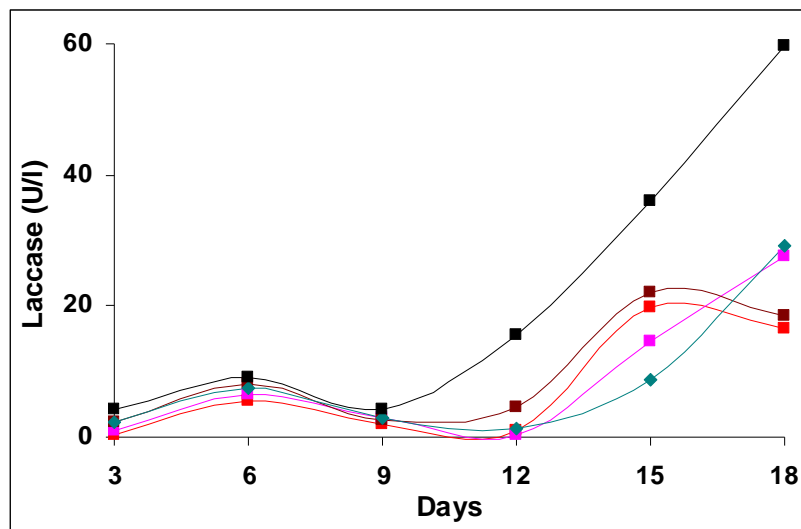


Figure 2.5 Laccase production by *Irpex lacteus* in the absence and presence of dye: Without dye (—); Remazol brilliant blue R (—); Reactive brown 18 (—); Effluent-1 (—); Effluent 3 (—).

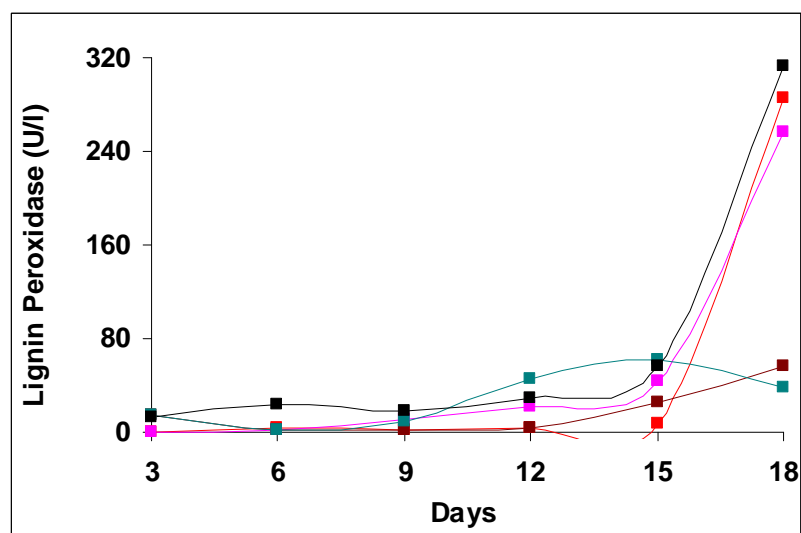


Figure 2.6 Lignin peroxidase production by *Irpex lacteus* in the absence and presence of dye: Without dye (—); Remazol brilliant blue R (—); Reactive brown 18 (—); Effluent-1 (—); Effluent 3 (—).

Irpex lacteus produced comparatively low ligninolytic activities in media containing effluents. *I. lacteus* produced higher ligninolytic activities in the medium containing remazol brilliant blue R supporting the results of Schliephake and Lonergan (1996) and Sollai *et al.*, (1996) where enhancement of extracellular activities in the presence of RBBR was observed. Marked increase in all ligninolytic activities after day 12 can be correlated with the extensive decolorization on and after day 12. These observations are similar to Sayadi and Ellouz (1993), who observed the contribution of ligninolytic activities in the decolorization of olive mill wastewater.

Irpex lacteus extensively decolorized different textile dyes and also complex textile effluents. The correlation between appearance of ligninolytic activities in the culture filtrate and initiation of decolorization can be attributed to the involvement of ligninolytic activities in the decolorization. The potential of *I. lacteus* could lead to the development of a more effective mycoremediation technique for decolorization of textile dyes and dye industry effluents.

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CHAPTER 3

SULFONPHTHALEIN DYE DECOLORIZATION BY LIGNINOLYTIC ENZYMES OF *Irpex lacteus* PRODUCED DURING SOLID-STATE FERMENTATION

3.1 Introduction

Interest in pollution potential of synthetic dyes used extensively in variety of industries has been prompted by concern over their possible toxicity and carcinogenicity. Bioremediation provides a cost-effective, eco-friendly and yet efficient technology. White rot fungi (WRF) are ubiquitous, having ability to degrade recalcitrant plant polymer lignin by its unique ligninolytic enzyme system. Non-specificity of ligninolytic enzymes permits WRF to degrade a wide variety of structurally diverse organopollutants (Hammel, 1992).

Most information on biodegradation of synthetic dyes by WRF has been obtained with *Phanerochaete chrysosporium* and few other species. The ability of *Irpex lacteus* to degrade xenobiotics is largely unknown; reports are available on decolorization of dyes (Novotny *et al.*, 2001). Sulfonphthalein dyes (pH indicators and tissue stains) are least studied with respect to fungal degradation. Purpose of this work was to study the production of ligninolytic enzymes by *I. lacteus* during solid-state fermentation of natural lignocellulose substrate, wheat straw, and decolorization of sulfonphthalein dyes by ligninolytic enzymes.

3.2 Materials and Methods

Microorganism

Irpex lacteus Fr. 238 617/93 obtained from Culture Collection of Basidiomycetes (CCBAS), Prague (Kind gift from V. Šašek).

Sulfonphthalein dyes

Sulfonphthalein dyes namely *o*-cresol red ($A_{577, 577} 41931 \text{ M}^{-1}.\text{cm}^{-1}$), phenol red ($A_{564, 564} 30737 \text{ M}^{-1}.\text{cm}^{-1}$), bromocresol green ($A_{621, 621} 39569 \text{ M}^{-1}.\text{cm}^{-1}$), bromophenol blue ($A_{583, 583} 52663 \text{ M}^{-1}.\text{cm}^{-1}$) and *m*-cresol purple ($A_{582, 582} 32406 \text{ M}^{-1}.\text{cm}^{-1}$) were used.

Production of ligninolytic enzymes by solid-state fermentation

The straw substrate flasks (250 ml Erlenmeyer) for solid-state fermentation were prepared as reported earlier (Vyas *et al.*, 1994) and inoculated with ten agar disks (8 mm) punched from actively growing cultures (5 day) on malt extract agar plates. The flasks were incubated at 28°C and harvested after 13 days when the entire content of the flasks was covered with mycelia. Extracellular enzyme extract was prepared according to Vyas *et al.*, (1994).

Enzyme assays and reactions

Manganese peroxidase (MnP), manganese-independent peroxidase (MIP), and laccase activities were determined as described earlier (Vyas *et al.*, 1994). Lignin peroxidase activity was estimated according to Tien and Kirk (1984). One unit of enzyme activity is defined as the amount of activity that will produce one μmol of the product per min upon oxidation of the substrate in the reaction mixture under assay conditions. Sulfonphthalein dye decolorization by MnP and MIP activities was assayed using reaction mixtures for MnP and MIP assays but the chromogen was replaced by respective sulfonphthalein dye. The reaction was terminated after 5 min with 125 μmol NaOH. Decolorization was followed at the λ_{max} of respective dyes. Dye decolorizing activity was calculated using molar absorption coefficient of the respective dye. One unit enzyme activity is defined as the amount of activity that consumes 1 μmol dye in the reaction mixture.

3.3 Results

Ligninolytic enzyme production

During solid-state fermentation of wheat straw *Irpex lacteus* produced ligninolytic activities of manganese peroxidase (232 U/l), manganese-independent peroxidase (106 U/l), lignin peroxidase (13 U/l) and laccase (16 U/l). Fig. 3.1 shows the influence of pH on the ligninolytic activities.

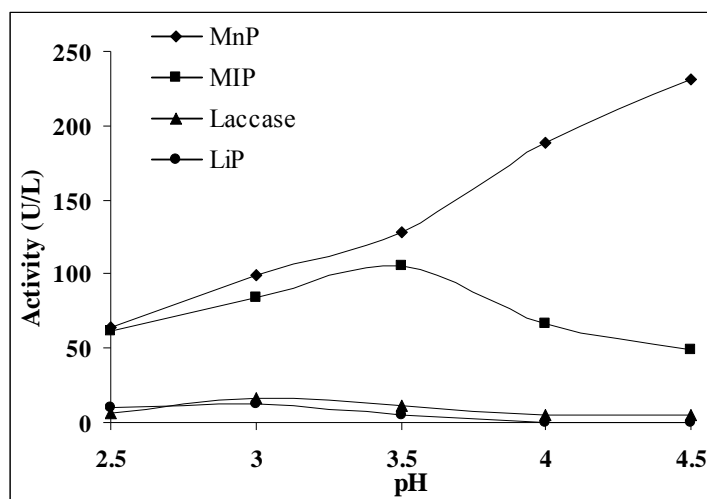


Figure 3.1 Influence of pH on ligninolytic enzymes produced by *Irpex lacteus* during solid-state fermentation of wheat straw

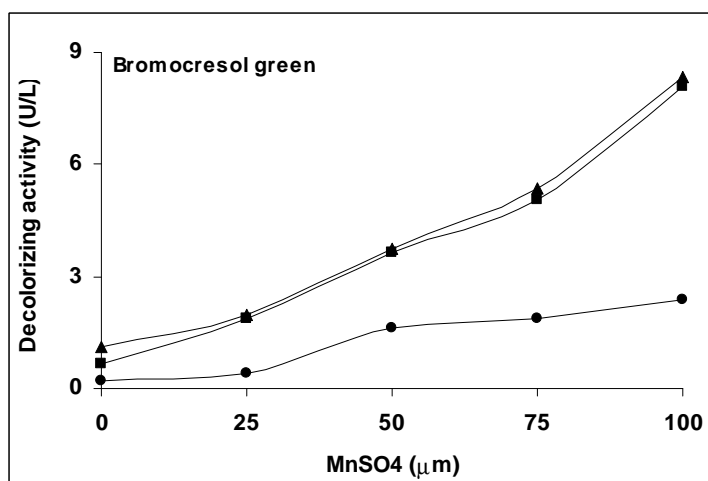
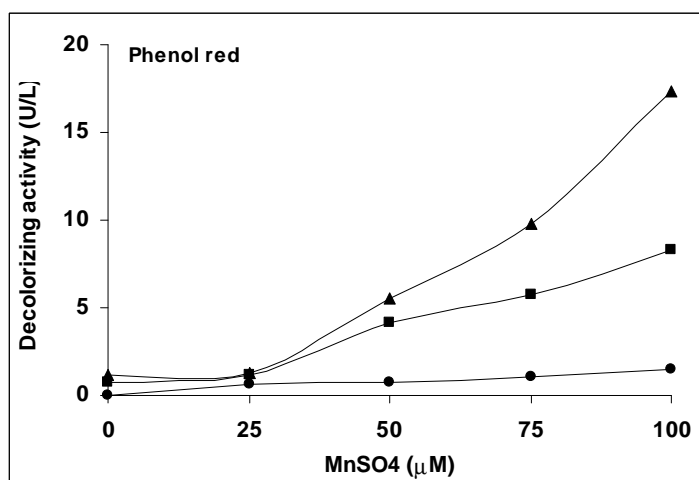
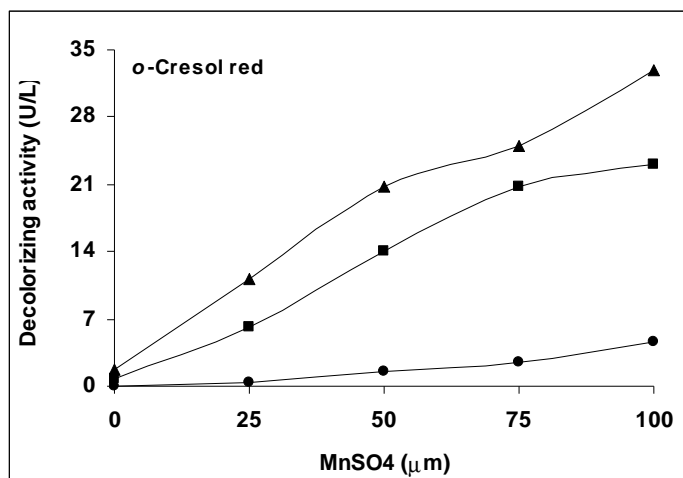
Decolorization of sulfonphthalein dyes

Decolorization of five different sulfonphthalein dyes with varying concentration of Mn^{2+} and pH was analyzed (Fig. 3.2). Decolorization of all the dyes except bromophenol blue increased with the increase in Mn^{2+} conc in the reaction mixture. This implies involvement of manganese peroxidase (MnP) in the dye decolorization reaction. MnP-catalyzed dye decolorization rates decreased with pH of the reaction mixture except in the case of bromocresol green where decolorization occurred equally well at pH 4-4.5. Only bromocresol green was decolorized at pH 3.5 albeit at lower rates by MnP.

Decolorization of *m*-cresol purple also occurred in the absence of Mn^{2+} indicating involvement of manganese-independent peroxidase (MIP) activity. In the pH range 4.0-4.5, increasing concentration of Mn^{2+} enhanced the decolorization rates whereas at lower pH the decolorization reaction was inhibited. Decolorization of *m*-cresol purple in the absence of Mn^{2+} occurred optimally at pH 3.5. Therefore decolorization of *m*-cresol purple at pH 4-4.5 is largely a function of MnP with a little contribution of MIP activity. At pH 3.5 *m*-cresol purple decolorization is solely attributed to MIP activity.

Bromophenol blue decolorization in the absence of Mn^{2+} and inhibition of the decolorization reaction upon subsequent increase in Mn^{2+} concentration suggests the role

of MIP activity in the decolorization, which occurred optimally at pH 4.5. Inhibition of enzymatic decolorization reaction by Mn^{2+} distinguishes MIP activity from other known ligninolytic activities.



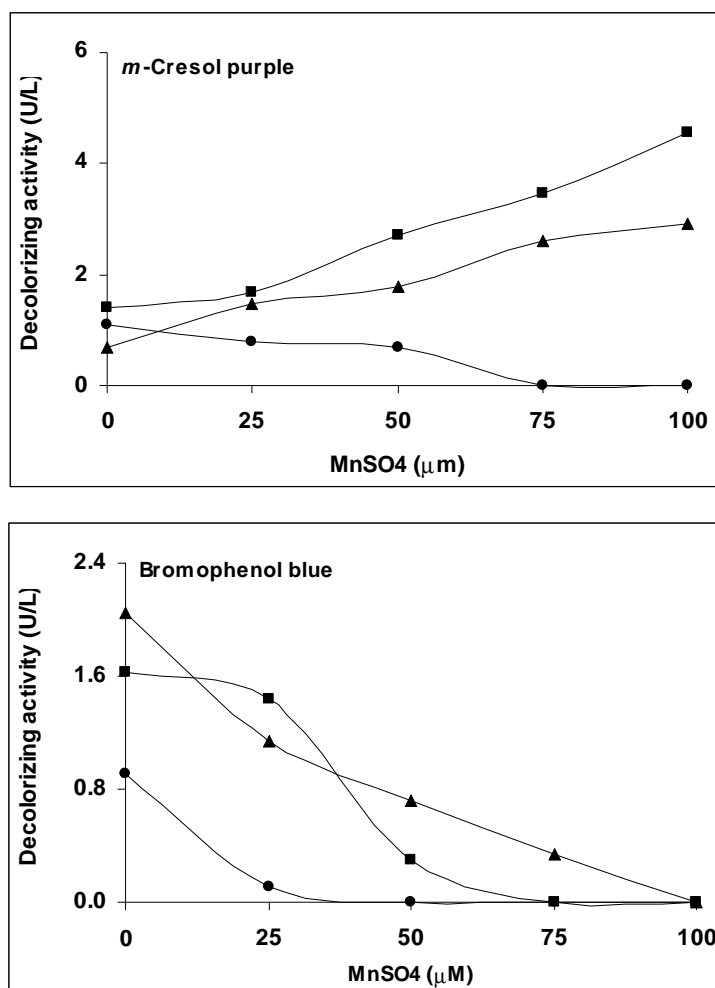


Figure 3.2 Influence of Mn(II) and pH [3.5 (●), 4.0 (○) and 4.5 (▲)] on decolorization of sulfonphthalein dyes by ligninolytic enzymes of *Irpex lacteus*

Influence of Inhibitors

Enzymatic dye decolorization reactions were inhibited to varying extent by thiourea, sodium metabisulfite, sodium azide and EDTA (Table 3.1).

3.4 Discussion

The extreme non-specificity and non-stereoselectivity of lignin degrading enzymes enables white rot fungi to degrade a broad spectrum of diverse aromatic pollutants. Vyas *et al.*, (1994) reported the production of ligninolytic enzyme during solid-state

fermentation of wheat straw and observed that all fungi studied did not produce all major ligninolytic activities under such near natural conditions.

Irpex lacteus under identical conditions produced manganese peroxidase (MnP), manganese-independent peroxidase (MIP), lignin peroxidase and laccase as observed with *Trametes versicolor* (Vyas *et al.*, 1994). Ligninolytic enzymes catalyze decolorization of synthetic dyes (Ollikka *et al.*, 1993, Christian *et al.*, 2003). Sulfonphthalein dyes contain free phenolic moiety that makes them better substrates of MnP as MnP attacks phenolic moieties while degrading lignin (Wariishi *et al.*, 1988).

MnP decolorizes *o*-cresol red, phenol red, bromocresol green and *m*-cresol purple but not bromophenol blue. Decolorization increased by increasing concentration of Mn^{2+} in the reaction mixture. However Mn^{2+} inhibited bromophenol blue decolorization; additional bromine groups on bromophenol blue chromophore impede oxidation of phenolic moiety and thereby making it a poor substrate for MnP. On the contrary additional Br groups improved the oxidizability of bromophenol blue by MIP. MIP activity is different from versatile peroxidase and lignin peroxidase since MIP-catalyzed dye decolorization reactions are inhibited in the presence of Mn^{2+} . Anthraquinone, azo and phthalocyanine dyes have been reported to be decolorized in a manganese-independent reaction (Vyas and Molitoris, 1995, Heinfling *et al.*, 1998).

Sodium metabisulfite, sodium azide and EDTA, a molecular oxygen scavenger, oxidase inhibitor and metal chelator respectively, inhibited MnP and MIP catalyzed sulfonphthalein dye decolorization reactions. This indicates that sulfonphthalein dye decolorization by MnP and MIP is an oxidative process requiring molecular oxygen and the presence and involvement of catalytic metal centre of the enzymes in the decolorization reaction. Hydroxyl radical is a strong oxidant and involved in wood decay (Hammel *et al.*, 1992). Inhibition of MnP-catalyzed decolorization by thiourea, a hydroxyl radical scavenger, indicates generation of hydroxyl radical by MnP and the role of hydroxyl radical in the decolorization reaction. Upon decolorization all sulfonphthalein dyes lose the pH indicator property attributing extensive degradation of dye chromophore by MnP and MIP activities (Table 3.1).

Dyes	% Inhibition			
	Thiourea (2.5 mM)	Na ₂ S ₂ O ₅ (2.5 mM)	NaN ₃ (2.5 µM)	EDTA (10 mM)
<i>o</i> -Cresol red ^a	22	44	28	99
Phenol red ^a	88	83	76	98
Bromocresol green ^a	100	70	25	93
<i>m</i> -Cresol purple ^a	16	68	23	76
Bromophenol blue ^b	0	72	85	83

^a MnP-catalyzed decolorization, ^b MIP-catalyzed decolorization

Table 3.1 Influence of inhibitors on enzyme catalyzed dye decolorization reactions

Irpex lacteus appears to be a potent organism for decolorization of dyes and degradation of other organopollutants as dye decolorizing ability is used as an indicator for screening the efficient organisms for pollutants degradation. Purification of these enzymes and degradation mechanism of dyes are presently being focussed.

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CHAPTER 4
LIGNINOLYTIC ENZYMES PRODUCTION AND
DECOLORIZATION OF SYNTHETIC DYES BY LITTER
BASIDIOMYCETE *Geastrum triplex*

4.1 Introduction

Water pollution control is presently one of the major areas of scientific activity. While colored organic compounds generally impart only a minor fraction of the organic load to wastewaters, their color renders them aesthetically unacceptable. Effluents discharged from textile and dyestuff industries to neighboring water bodies and wastewater treatment system is of significant health concern. These dyes include several structural varieties, such as acidic, reactive, basic, disperse, azo, diazo, anthraquinone-based and metal-complex dyes which are used for various industrial applications including dyestuffs, textile, paper and leather industries. Conventional wastewater treatment is not efficient to remove recalcitrant dyestuffs from effluents (Shaul *et al.*, 1991). Despite the existence of a variety of processes, bioremediation is still seen as an attractive solution due to its reputation as a low-cost, eco-friendly and publicly acceptable treatment technology (Banat *et al.*, 1996).

Mostly bacterial system is used for the bioremediation. Bioremediation may also involve lignin degrading basidiomycetous fungi, characterized by their ability to degrade lignin and has made them an important link in the global carbon cycle (Kirk and Farrell, 1987). White rot fungi have been extensively studied and shown to degrade a wide range of persistent chemicals in both liquid and soil (Barr and Aust, 1994). The redox potentials of LiP and MnP are higher than for most other peroxidases. This allows these enzymes to oxidize of chemicals that are not easily oxidized.

Most of the work related to ligninolytic enzymes and decolorization of dyes has been concentrated on selected white rot basidiomycetes. Our approach was to study the efficiency of unexplored litter basidiomycetes for the production of ligninolytic enzymes and analyze their potential in the decolorization of synthetic dyes.

4.2 Materials & methods

Organism

Litter basidiomycete *Geastrum triplex* was isolated from fruiting body collected from Kathiyawad peninsula, Gujarat, India.

Dye decolorization on solid media

The plates of malt extract agar containing sulfonphthalein dyes (10⁻⁵ M) bromophenol blue and phenol red, triphenylmethane dyes (10⁻⁵ M) fast green and methyl violet, textile dyes (0.1%) reactive blue H5G and reamzol magenta HB were inoculated with a disc punched from actively growing cultures of *G. triplex* on malt extract agar plates and incubated at room temperature. The plates were observed daily for the zone of growth and dye decolorization.

Production of ligninolytic enzymes

Shallow stationary cultures of *G. triplex* were grown on 10 ml modified Kirk's medium in 250 ml Erlenmeyer flasks. Peptone (0.5 g l⁻¹), yeast extract (0.1 g l⁻¹) and acetate buffer (100 mM, pH 4.2) were used instead of ammonium tartarate and dimethyl succinate buffer. 10 agar discs punched from actively growing cultures on malt extract agar plates were used to inoculate the medium. The flasks were incubated at room temperature and harvested at three days intervals, starting from 3rd to 18th day. The harvest was filtered, and spun (8000 rpm, 15 min, 4°C). Supernatant was collected and analyzed for enzymatic activities.

Biochemical analysis

MnP activity was determined spectrophotometrically by measuring oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) + DMAB (3-dimethylamino benzoic acid) as chromogen according to Vyas *et al.*, (1994). One unit of enzyme activity is defined as the activity that produces one μ mol of the colored product per min upon oxidation of MBTH-DMAB in the above reaction mixture. Lignin peroxidase activity was assayed according to Tien and Kirk (1984). Unit enzyme activity is defined as the amount of activity that will produce 1 μ mol of veratraldehyde per min upon oxidation of veratryl alcohol at room temperature.

Sulfonphthalein dye decolorization by ligninolytic enzymes was assayed as reported earlier (Christian *et al.*, 2003). In the case of triphenylmethane and textile dyes, DMAB + MBTH or veratryl alcohol was replaced by respective dyes. Reactions at different pH were performed using succinate lactate buffer (0.1 M, pH 4.0, 4.5), tartrate buffer (0.1 M, pH 2.5, 3.0, 3.5). All reactions were performed at room temperature. Unit enzyme activity is defined as the amount of activity that will consume 1 μmol of dye per min in the above reaction mixture. Molar absorption coefficient of the dye was used to compute enzymatic activities. In the case of textile dyes unit enzyme activity is defined as the amount of activity that will cause the change of 1 AU per min in the reaction mixture.

4.3 Results

Isolation of litter basidiomycetes

Fruiting body of the litter basidiomycete was collected from Kathiawad Peninsula and identified as *Geastrum triplex* (Dr. J. G. Vaidya, Personal communication) that belongs to class Gasteromycetes and order Lycoperdales (Puffballs). *Geastrum triplex* is probably the commonest earthstar but still infrequently found. This species usually occurs in woodland litter. A part of fruiting body was cut, surface sterilized and plated on water agar. Mycelia sprouting from the fruiting body were transferred on malt extract agar plates. The culture was obtained in pure by serial transfers on the same medium.

Dye decolorization on solid media

Geastrum triplex completely decolorized the dyes added to the agar medium within 14 days. Decolorization initiated from the place where the disc was placed and it lagged behind the zone of growth. Decolorization of methyl violet and phenol red initiated after 2 days and were decolorized completely within 7 days while fast green and bromophenol blue were decolorized within 9 days. Decolorization of the two textile dyes initiated after 9 days; complete decolorization of the dyes in the medium occurred within 2 days after initiation of decolorization.

Production of ligninolytic enzymes by shallow stationary cultures

Shallow stationary cultures of *G. triplex* produced extracellular manganese peroxidase (MnP), lignin peroxidase (LP) and laccase activities (Fig. 4.1) as early as day 3 and through out the experimental run (day 18). Production of MnP under the experimental conditions peaked on day 6 and also gave a smaller peak on day 15 before decreasing. LiP activity was highest on day 3 before decreasing and later on did not change much while laccase activity peaked on day 15 before decreasing. Production of MnP was higher than the other two activities.

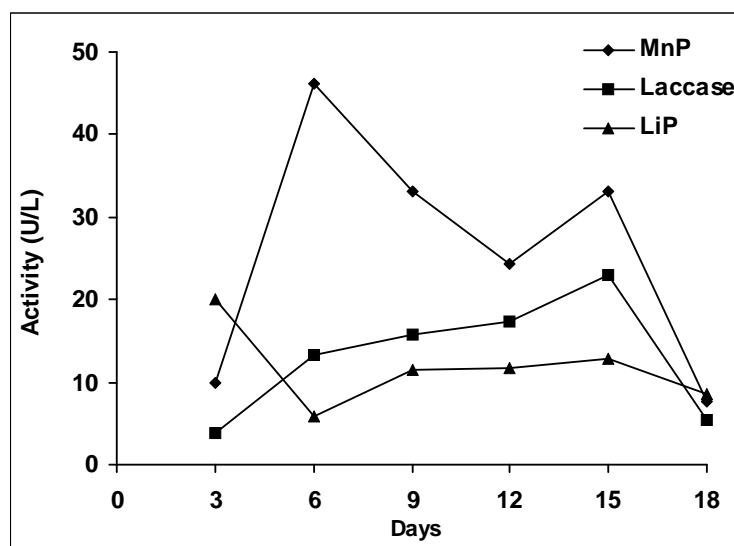


Figure 4.1 Ligninolytic activities produced by *Geastrum triplex*

Decolorization of synthetic dyes by ligninolytic enzymes of Geastrum triplex

Decolorization of phenol red, reactive blue H5G, remazol magenta HB and fast green by MnP activity occurred best at pH 4. The decolorization rates were marginally inferior at pH 3.5. MnP activity did not decolorize fast green and methyl violet at pH 3.5. Decolorization of phenol red, reactive blue and remazol magenta improved with the increase of Mn(II) conc in the reaction mixture at all the pHs tested (Fig. 4.2). MnP-catalyzed methyl violet decolorization occurred optimally at pH 4.5. The MnP and LiP activity profiles vary significantly for different dyes and the substrates used for its estimation (Fig. 4.3, 4.4). Kinetic constants of all dyes for MnP and LiP are shown in Table 4.1.

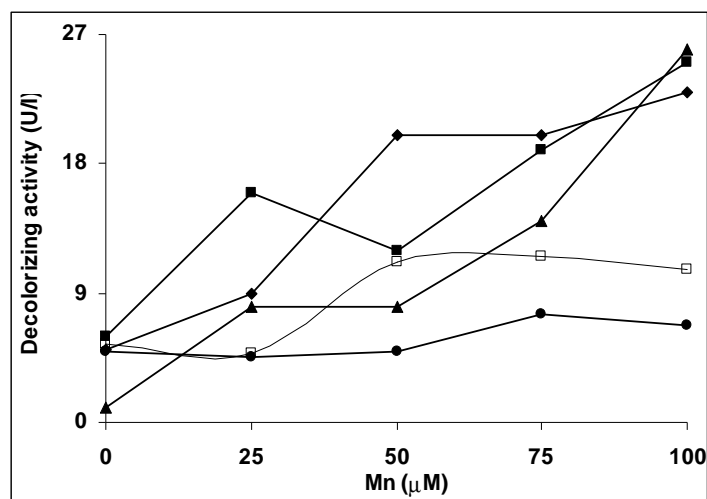


Figure 4.2 Influence of Mn(II) on decolorization of phenol red (\square), methyl violet (\bullet), remazol magenta HB (\circ), reactive blue H5G (f) and fast green (i) by manganese peroxidase activity produced by *Geastrum triplex*

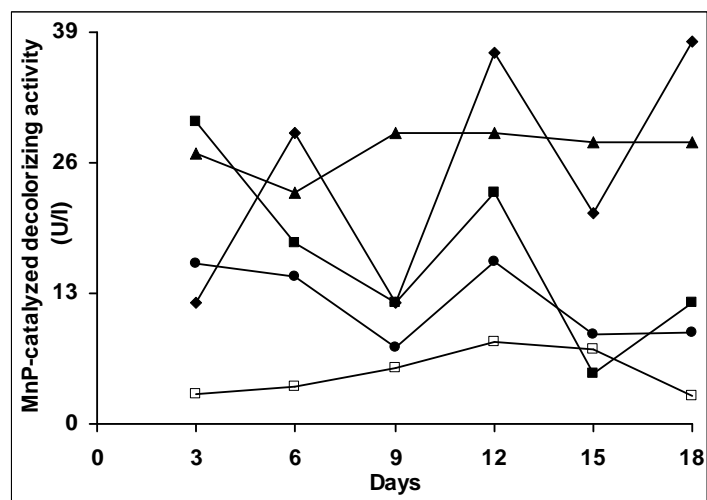


Figure 4.3 Decolorization of phenol red (\square), methyl violet (\bullet), remazol magenta HB (\circ), reactive blue H5G (f) and fast green (i) by manganese peroxidase activity produced by *Geastrum triplex*

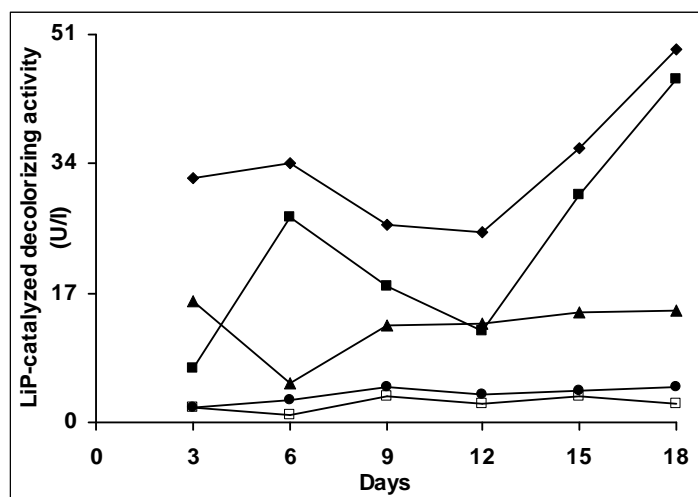


Figure 4.4 Decolorization of bromophenol blue (□), methyl violet (●), remazol magenta HB (△), reactive blue H5G (▽) and fast green (◇) by lignin peroxidase activity produced by *Geastrum triplex*

These activities produced on different days exhibited variation in their ability to use the dyes as the substrate. Bromophenol blue, reactive blue, remazol magenta, methyl violet and fast green were decolorized by LiP. Decolorization rates increased concomitantly with the increase in the concentration of veratryl alcohol (VA) in the reaction mixture (Fig. 4.5). Optimal pH for the LiP-catalyzed decolorization of the dyes was not similar.

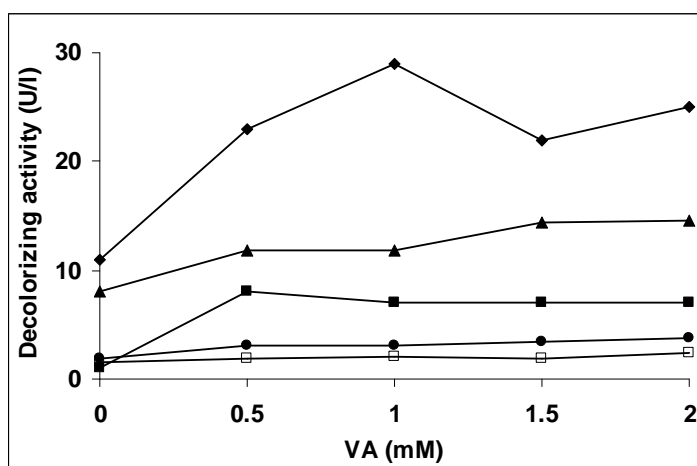


Figure 4.5 Influence of veratryl alcohol on decolorization of bromophenol blue (□), methyl violet (●), remazol magenta HB (△), reactive blue H5G (▽) and fast green (◇) by lignin peroxidase activity produced by *Geastrum triplex*

Dyes	MnP		LiP	
	K_m (μ M)	V_{max} (U/ml)	K_m (μ M)	V_{max} (U/ml)
Phenol Red	500	0.076	-	-
Bromophenol blue	-	-	0.641	0.018
Remazol magenta	27.02	0.014	0.909	0.041
Reactive blue	142.85	0.058	0.2	0.045
Fast green	125	0.028	2.02	0.003
Methyl violet	23.25	0.008	0.769	0.007

Table 4.1 Kinetics constants of sufophthalein, textile and triphenylmethane dyes for manganese peroxidase and lignin peroxidase activity produced by *Geastrum triplex* during shallow stationary cultures growing on GVT medium.

4.4 Discussion

The extracellular, non-specific and highly oxidative nature of the ligninolytic enzymes produced by lignin degrading white rot basidiomycetes confer these organisms with a unique ability to degrade a wide range of organopollutants (Hammel, 1992; Barr and Aust, 1994). This has generated a great deal of interest in using this group of fungi in pollution abatement.

Information pertaining the production of ligninolytic enzymes that is available has been largely obtained with *Phanerochaete chrysosporium* and to a certain extent with few other selected white rot basidiomycetes. The need to screen a large number of lignin degrading basidiomycetes for their potential to degrade xenobionts has been emphasized by several investigators (de Jong *et al.*, 1992; Orth *et al.*, 1994; Banat *et al.*, 1996). Paucity of information about the xenobiotic and lignin degrading abilities of litter basidiomycetes prompted us to undertake the isolation and identification of litter basidiomycetes. *Geastrum triplex* is one of the several litter basidiomycetes strains isolated in our laboratory.

During the early stage of development, the fruit body of *G. triplex* is more or less spherical but as it matures the outer part (the periderm) splits open to form the characteristic rays. The expansion of the rays helps to lift the fruit body clear of the litter layer. Cultures of *G. triplex* growing on semi solid media decolorized sulfonphthalein, triphenylmethane and textile dyes. Decolorization occurred only under old mycelium like in case of wood rotting white rot basidiomycetes.

The litter basidiomycete *G. triplex* was found to produce ligninolytic activities manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase activities under nutrient limiting conditions. Unlike lignin degrading white rot basidiomycetes, *G. triplex* produced LiP activity much sooner and concomitantly with the MnP and laccase activities.

The MnP activity of *G. triplex* decolorized phenol red, reactive blue H5G, remazol magenta HB and fast green but not bromophenol blue. Decolorization occurred over the tested pH range (4.5 to 3.5). Decolorization of triphenylmethane dyes methyl violet and fast green by MnP was poor. MnP activity of *G. triplex* resembles the MnP activity of white rot basidiomycetes in its substrate specificity (Christian *et al.*, 2003).

LiP activity decolorized sulfonphthalein, triphenylmethane and textile dyes. Reactive blue, remazol magenta and bromophenol blue were more favorable for decolorization by LiP activities. Veratryl alcohol perhaps serves as the mediator in LP-catalyzed decolorization reaction as implied by the direct correlation observed between decolorization rate and veratryl alcohol concentration.

MnP and LiP exhibited overlapping substrate specificities in decolorizing triphenylmethane and textile dyes but varied in using sulfonphthalein dyes. MnP did not decolorize bromophenol blue while LiP that decolorized bromophenol blue did not decolorize phenol red which was found to be the best substrate for MnP. Such dyes can be used to distinguish these activities.

Differences in the decolorization profiles imply that the isozyme composition representing MnP and LiP activities changes with time. Similar observations have been reported with *Phanerochaete chrysosporium*, *Trametes versicolor*, *Phlebia radiata* and

Pleurotus ostreatus (Farrell *et al.*, 1989; Johansson and Nyman, 1993; Vares *et al.*, 1993; Martinez *et al.*, 1996).

The litter basidiomycete *G. triplex* possesses the ability to decolorize structurally diverse dyes. This ability is attributed to the ligninolytic enzymes MnP and LiP of *G. triplex*. Further work needs to be done, to improve production of ligninolytic enzymes by manipulating medium composition, to purify and characterize these activities, to investigate the ability of *Geastrum triplex* to proliferate in soil and its ability to degrade such pollutants in soil.

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CHAPTER 5

MEDIATOR ROLE OF VERATRYL ALCOHOL IN THE LIGNIN PEROXIDASE-CATALYZED OXIDATIVE DECOLORIZATION OF REMAZOL BRILLIANT BLUE R

5.1 Introduction

White-rot fungi (WRF) play a central role in global carbon cycle as a result of their innate ability to mineralize the woody plant material lignin, which has a complex polymeric structure. The WRF appear to be unique in their ability to degrade lignin by the secretion of relatively non-specific, highly oxidative, extracellular ligninolytic system. Ligninolytic system of WRF consists of a pool of enzymes, namely lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, laccase, cellobiose dehydrogenase, and H₂O₂-producing enzymes.

The ligninolytic enzymes of WRF are highly non-specific and have been implicated in the transformation and mineralization of organopollutants having structural similarities with lignin (Aust, 1990; Cameron *et al.*, 2000; Ponting, 2002). Ligninolytic cultures of several white rot fungi have been reported to degrade and decolorize various dyes (Shah and Nerud, 2002; Wilkolazka *et al.*, 2002; Christian *et al.*, 2003). Involvement of MnP and LiP has been demonstrated in the degradation pathway of some of the dyes (Paszczyński and Crawford, 1991; Spadaro and Renganathan, 1992).

LiP, a heme-containing glycoprotein has an unusually low pH optimum is able to catalyze the oxidation of variety of compounds with high reduction potential. In nature, lignin peroxidase can oxidize both phenolic and non-phenolic lignin related compounds resulting in cleavage of the C_α-C_β bond, the aryl-C bond, aromatic ring opening, phenolic oxidation and demethoxylation. Due to its high redox potential and enlarged substrate range in the presence of specific mediators LiP has great potential for application in various industrial processes.

Veratryl alcohol (VA) enhances the action of LiP on many substrates, including lignin (Gold *et al.*, 1989), by acting as a mediator (Hammel *et al.*, 1993), or by protecting the enzyme against inactivation (Harvey *et al.*, 1986). LiP catalyzes oxidation of VA to VA cation radical (VA^{•+}), which is a powerful charge-transfer reagent that can oxidize large hydrophobic molecules like lignin and other recalcitrant molecules by indirect oxidation (Wariishi and Gold, 1989).

Remazol brilliant blue R (RBBR), an industrially important dye is an anthracene-derivative and represents an important class of often toxic and recalcitrant organopollutants. It structurally resembles certain polycyclic aromatic compounds, which are substrates of ligninolytic peroxidases (Khindaria *et al.*, 1995; Hammel, 1992). RBBR is decolorized by LiP and veratryl alcohol reportedly improves LiP-catalyzed RBBR decolorization (Ollikka *et al.*, 1993). Our present report deals with the experimental assessment of the role of VA in RBBR-decolorization by LiP and its implications.

5.2 Materials and methods

Chemicals

Remazol brilliant blue R (RBBR) (Sigma), 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), and 3-dimethylaminobenzoic acid (DMAB) (Lancaster) were used.

Microorganisms

Trametes versicolor was maintained on malt agar slants at 5°C. Transfers were made on malt agar plates and cultivated at 28°C.

Production and preparation of extracellular enzyme extract

Solid-state fermentation of wheat straw by *Trametes versicolor* was carried out as described earlier (Vyas *et al.*, 1994a). A set of 5-7 flasks was harvested at various time points and immediately processed for the preparation of extracellular enzyme extract as described earlier (Vyas *et al.*, 1994a). The concentrate was spun (5°C, 3 min) before using it for biochemical analyses. Shallow stationary cultures of *T. versicolor* growing on modified Kirk's medium were set up. Peptone (0.5 g.l⁻¹) and yeast extract (0.1 g.l⁻¹) were

added instead of ammonium tartarate and acetate buffer was used instead of dimethyl succinate buffer. A set of 5-7 flasks was harvested on 12th day. Extracellular fluid was desalted and concentrated by ultrafiltration, before using it for biochemical analyses.

Biochemical analyses

Manganese peroxidase (MnP), manganese-independent peroxidase (MIP), and laccase activities were determined as described Vyas *et al.*, (1994b). Lignin peroxidase (LiP) activity was estimated according to Tien and Kirk (1984). One unit of enzyme activity is defined as the amount of activity that will produce one μmol of the product per min upon oxidation of the substrate in the reaction mixture (RM).

RBBR-decolorizing enzyme activity was assayed as reported earlier (Vyas and Molitoris, 1995). RBBR and VA were added, where indicated, as aqueous solutions to the RM, to give final concentrations as indicated in the legends to the respective figures. Reaction was initiated by adding H_2O_2 .

5.3 Results

*Ligninolytic activities produced by *Trametes versicolor**

Trametes versicolor produced various ligninolytic activities during solid-state fermentation of wheat straw. While other activities were produced as early as on day 3, lignin peroxidase activity was detectable in the 8-day sample and onwards (Table 5.1).

RBBR decolorizing activity

RBBR-decolorizing activity was also detected in 3- and 5- day samples (data not shown here) but it was very low. This activity was H_2O_2 -dependent, and independent of Mn^{II} and was not influenced by the presence or absence of veratryl alcohol. RBBR-decolorizing activity increased several-fold in the 8 and 13 day samples during solid state fermentation of wheat straw and shallow stationary cultures on N-limited medium (Table 5.2). This increase in activity was associated with the appearance of LiP activity.

Ligninolytic activity	Incubation time	Activity
	(Days)	(U/g dry wight straw)
MnP	3	0.024
MIP	3	0.202
Lac	3	0.012
LP	8	0.333
RBBR-decolorizing	3	0.011
RBBR-decolorizing	8	0.063 ^a
	8	0.123 ^b

^ain the absence of veratryl alcohol, ^bin the presence of veratryl alcohol

Table 5.1 Ligninolytic activities produced by *Trametes versicolor* during solid state fermentation of wheat straw

pH	RBBR decolorizing lignin peroxidase activity		
	SSF 8	SSF 13	SSC 12
	(U/g dry wt straw)	(U/g dry wt straw)	(U/ml)
2.5	0.035	0.091	nd
3.0	0.188	0.220	0.826
3.5	0.226	0.215	1.069
4.0	0.190	0.176	0.981
4.5	0.140	0.080	nd

nd- not determined

Table 5.2 Influence of pH on RBBR decolorization by lignin peroxidase activity produced by *Trametes versicolor* during solid state fermentation of wheat straw (8th and 13th Day) and shallow stationary cultures growing on low-N medium (12th Day).

Influence of veratryl alcohol on RBBR decolorizing activity

RBBR was decolorized by LiP activity produced by the cultures of *T. versicolor* growing on wheat straw and low-N medium. Presence of VA for RBBR decolorization was not mandatory but VA improved RBBR decolorization rates. K_M for RBBR and VA of LiP-catalyzed RBBR decolorization are shown in Table 5.3.

pH	<i>K_m</i>			
	RBBR (μM)		VA (μM)	
	8 th Day	13 th Day	8 th Day	13 th Day
2.5	35	88	46	222
3.0	1754	141	40	255
3.5	154	86	55	198
4.0	93	69	76	431
4.5	48	29	40	153

Table 5.3 K_m for RBBR and veratryl alcohol of lignin peroxidase activity at various pH.

Addition of VA increased and stabilized RBBR decolorization rates at all pH tested. Increases of VA conc (> 500 μM) terminated reaction sooner and temporarily reversed the reaction. Rates of reverse reaction were higher with the higher VA concentrations. Enzymatic decolorization of RBBR decolorization by 8 and 13 day sample in the absence of VA occurred optimally at pH 3.5 and 3.0 respectively whereas enhancement of RBBR decolorization by VA optimally at pH 4.0 (Fig. 5.1).

Up to 300 μM VA concentration, RBBR-decolorizing activity increased, however, with higher VA concentrations (> 500 μM) it did not increase proportionally and levelled-off exhibiting saturation kinetics. (Fig. 5.1).

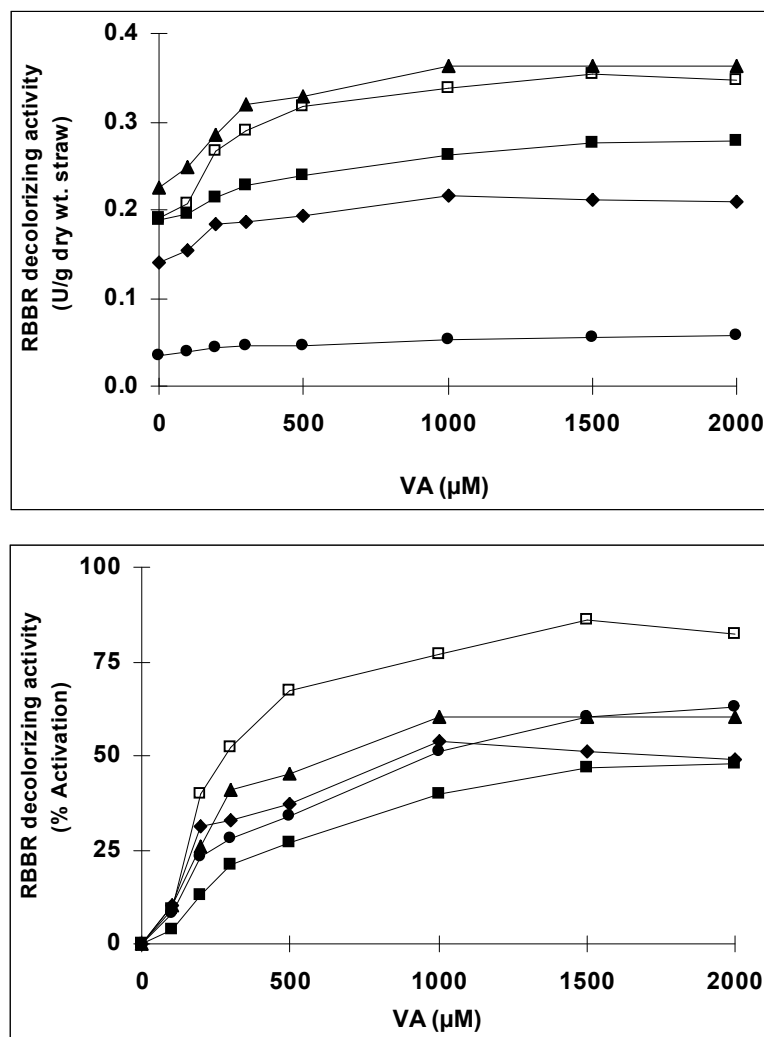


Figure 5.1 Influence of veratryl alcohol on RBBR decolorization at different pH [2.5 (●), 3.0 (□), 3.5 (■), 4.0 (△), 4.5 (◆)] by lignin peroxidase activity produced by *Trametes versicolor* during solid state fermentation of wheat straw (8th Day).

Influence of RBBR on veratryl alcohol oxidation and RBBR decolorization by LiP

RBBR-decolorization by LiP activity of *T. versicolor* growing on GVT medium (12 day) had an optimum pH of 3.0 and it improved with increases of RBBR in the reaction mixture and at the same time decreased VA oxidizing LiP activity (Table 5.4). VA was readily oxidized to veratryl aldehyde in the absence of RBBR. However, addition of RBBR introduced a lag in the formation of veratryl aldehyde from VA. Increases in RBBR concentration increased the lag period and also decreased the rate of formation of veratryl aldehyde (Fig 5.2).

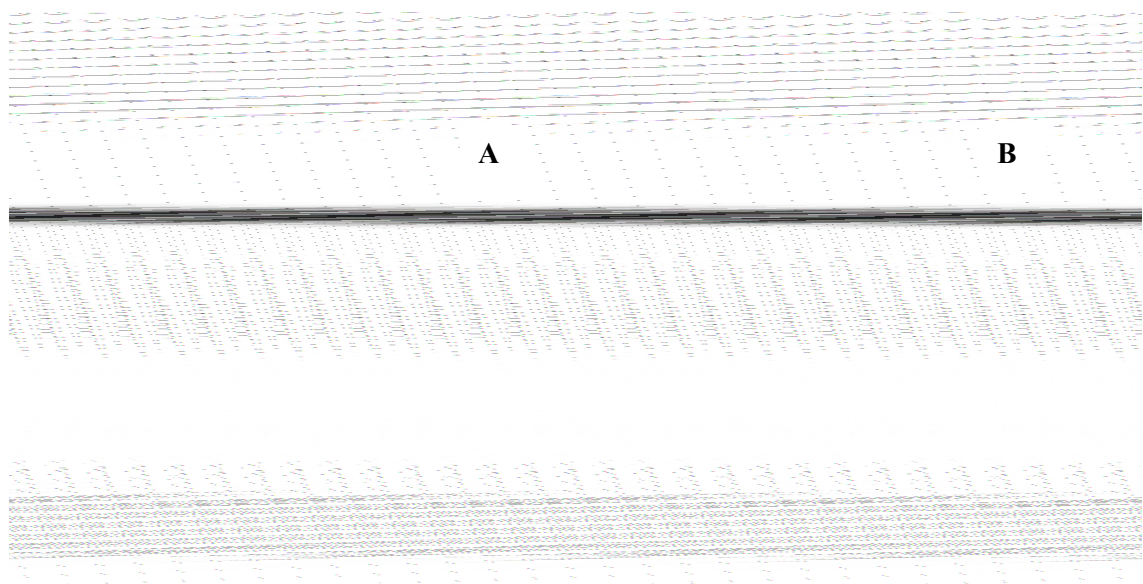


Figure 5.2. Influence of RBBR **(A)** [0 μM (1), 12.5 μM (2), 25 μM (3) and 50 μM (4)], **(B)** [12.5 μM (1), 25 μM (2), 50 μM (3) and 100 μM (4)] on veratryl alcohol oxidation by lignin peroxidase activity produced by *Trametes versicolor* **(A)** growing on N-limited medium (12th Day), **(B)** during solid state fermentation of wheat straw (8th Day).

RBBR (μM)	Lignin peroxidase activity	
	RBBR decolorization	Veratryl alcohol oxidation
	(U/g dry wt straw)	(U/g dry wt straw)
12.5	0.029	0.096
25	0.081	0.082
50	0.191	0.045
100	0.161	0.013

Table 5.4 Influence of RBBR on RBBR decolorization and veratryl alcohol oxidation by lignin peroxidase produced by shallow stationary cultures of *Trametes versicolor* (12th Day).

Influence of RBBR on RBBR decolorization by LiP

As the concentration of RBBR increases amount of VA required for 50% activation of RDA by LiP also increases. As the ratio of VA:RBBR decreases in activation of RBBR-decolorizing activity (%) by VA also decreases. At RBBR concentration (150 μM) in the reaction mixture, VA fails to significantly improve RBBR-decolorizing activity (Fig. 5.3).

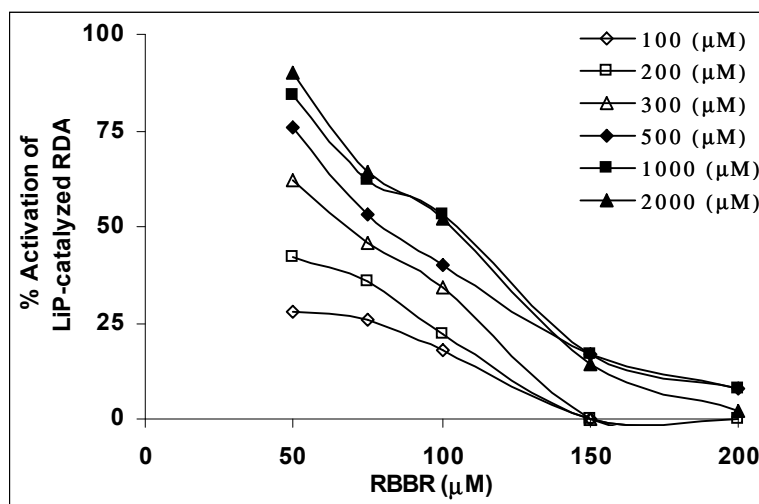


Figure 5.3 Influence of RBBR at various concentrations of veratryl alcohol on RBBR decolorization by lignin peroxidase activity produced by *Trametes versicolor* during shallow stationary culture on N-limited medium (12th Day)

5.4 Discussion

Anthraquinone-based dyes are structurally similar to the lignin backbone and are thus efficiently decolorized by the white rot fungi. Remazol brilliant blue R (RBBR) is being used for measuring ligninolytic activity by several investigators (Vyas and Molitoris, 1995; Novotny *et al.*, 2001; Thorne, 1993). RBBR has been shown to be decolorized by lignin peroxidase (LiP) of *Phanerochaete chrysosporium* (Ollikka *et al.*, 1993) and an extracellular oxygenase (ligninolytic) activity of *Pleurotus ostreatus* (Vyas and Molitoris, 1995). Recently Moreira *et al.*, (2001) described decolorization of RBBR by a peroxidase that oxidize Mn^{II} as well as veratryl alcohol (VA) and 2,6-dimethoxyphenol in a manganese-independent reaction.

The white-rot fungus *Trametes versicolor* is a preferential lignin degrader and has been shown to degrade PCB 77, anthracene and other xenobiotic compounds including certain dyes (Vyas *et al.*, 1994c; Vyas *et al.*, 1994d; Freitag *et al.*, 1992; Knapp *et al.*, 1995). It decolorizes RBBR during its growth on low N medium. RBBR decolorizing activity of *T. versicolor* is extracellular and is produced as a part of ligninolytic enzyme system.

Decolorization of RBBR by *T. versicolor* involves LiP activity and an H₂O₂-dependent activity which is not influenced by VA and Mn^{II}. RBBR decolorization however, was observed largely a function of LiP during the later stages of growth.

LiP activity of *T. versicolor*, like that of *Phanerochaete chrysosporium*, possesses the ability to decolorize RBBR and uses it as its substrate (Ollikka *et al.*, 1993). RBBR can also be used as a substrate for the detection and estimation of LiP activity. Improvement of RBBR decolorization rates in the presence of VA suggests that VA serves as a mediator in the LiP-catalyzed RBBR decolorization. It was also observed that the pH at which LP activity is maximal, is also optimal for RBBR-decolorizing activity. VA activated the RBBR decolorizing activity by 70-100 % with pH optimum of 4.0. VA has been found to enhance the rate and extent of chemical oxidation by LiP activity (Barr and Aust, 1994a; Chung and Aust, 1995a; 1995b). Here, VA can serve as an electron mediator to facilitate the oxidation of RBBR.

We evaluated mediator role of VA in RBBR decolorization by following simultaneously VA oxidation and RBBR decolorization by LiP. It was observed that increases in RBBR concentration increased the decolorization rates; however LiP activity measured as a function of formation of veratryl aldehyde, decreased and appeared after a lag period. The lag period increased with increases in RBBR concentration. This suggests that RBBR had better reactivity than VA for LiP. The lag in LiP activity in the presence of lower (> 12.5 µM) concentrations increased at 25 µM RBBR. But at 50 µM RBBR, LiP activity occurred slowly without showing any lag. During this period, RBBR-decolorizing activity had ended or rates had diminished. Although the concentration of VA was quite higher than RBBR, the oxidation of RBBR preceded oxidation of VA. This indicates that RBBR compete with VA and is the preferred substrate, giving a lag period preceding veratryl

aldehyde formation. Similar veratryl alcohol mediated oxidation was observed with pentachlorophenol (Chung and Aust, 1995b).

The other reason for this phenomenon is that VA cation radical ($VA^{\bullet+}$) generated upon oxidation of VA decolorized RBBR and getting reduced back to VA, giving the appearance of inhibition of VA oxidation. The inhibitory influence may also be due to the conversion of LiP into compound III, an inactive intermediate in the presence of higher RBBR concentration as observed in the case of PCP oxidation by LiP (Chung and Aust, 1995b). $VA^{\bullet+}$ that is responsible for the conversion of compound III to ferric enzyme (Barr and Aust, 1994b) could not protect the enzyme against inactivation as (i) $VA^{\bullet+}$ generation decreases in the presence of RBBR and (ii) $VA^{\bullet+}$ generated are consumed in RBBR oxidation. Our results thus support the speculation of Chung and Aust (1995a) that $VA^{\bullet+}$ consumed in the indirect oxidation of RBBR (other chemicals) leads to the loss of enzyme activity.

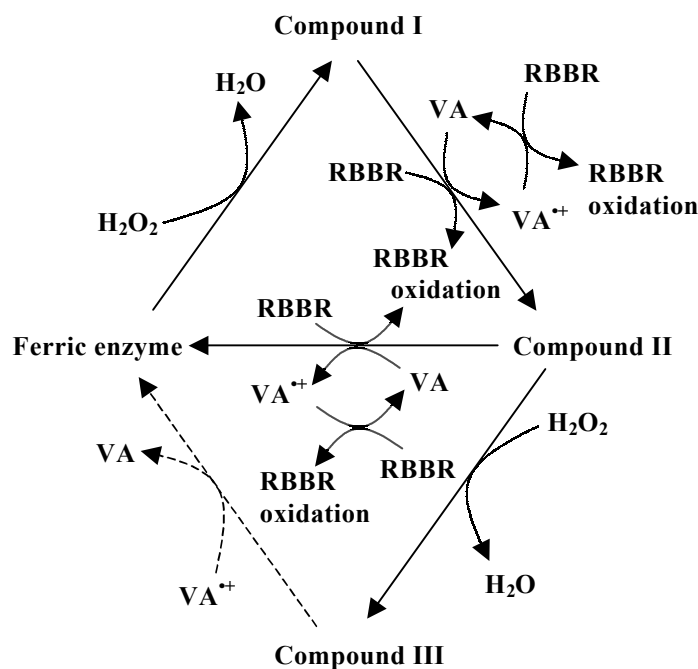


Figure 5.4 Mechanism showing role of veratryl alcohol in the decolorization of RBBR by lignin peroxidase activity

In the presence of higher concentrations of RBBR ($100\text{ }\mu\text{M}$) the proportional activation upon increasing concentration of VA becomes less significant and RBBR decolorizing activity occurred independent of VA. This is suggestive of (i) direct oxidation of RBBR by LiP and (ii) competitive inhibition of LiP-catalyzed VA oxidation by RBBR.

Strong evidences for the mediator role of VA in the LiP-catalyzed oxidation of RBBR (Fig. 5.4) are observed in the facts i) improvement of RBBR decolorization rates by VA, 2) stabilization of RBBR decolorization reaction by VA, iii) inhibition of veratryl aldehyde formation by RBBR and iv) inhibition of LiP activity is a function of RBBR concentration.

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CHAPTER 6
DECOLORIZATION OF SULPHONPHTHALEIN DYES BY
MANGANESE PEROXIDASE ACTIVITY OF THE WHITE ROT
FUNGUS *Phanerochaete chrysosporium*

6.1 Introduction

Dyes constitute an important family of organopollutants since they are an essential part of our civilization and represent structurally and chemically diverse group of xenobiotic compounds. Dyes are recalcitrant compounds; some of them are toxic and even carcinogenic. Synthetic dyes and pigments, released into the environment mainly in the form of wastewater effluents by textile, dyeing, printing, food and leather industries, cause severe ecological problems (Nerud *et al.* 2001). Between 10 and 15% of the total dye consumed in dyeing processes may be found in wastewater (Spadaro *et al.*, 1992). Physical and chemical methods used for removal of dyes i.e. adsorption, chemical transformation, incineration, photocatalysis or ozonation, are effective but rather costly (Banat *et al.*, 1996). Dyes being highly resistant to microbial attack are hardly removed from effluents by conventional biological wastewater treatment such as activated sludge (Shaul *et al.*, 1991). There is a need to develop a practical biological method of dye waste treatment that can be used for a wide range of wastes.

The potent biological system involves, white rot fungi, a group of basidiomycetes characterized by their ability to degrade and mineralize lignin completely in wood (Zabell and Morell, 1992). Free radical based, unique nonspecific lignin degrading system of white rot fungi has made them effective over a variety of hazardous environmental pollutants including various dyes (Glenn and Gold, 1983; Bumpus *et al.*, 1985; Hammel, 1992; Barr and Aust, 1994; Paszczynski and Crawford, 1995). The ability of these fungi to degrade lignin and structurally diverse pollutants results from the relatively nonspecific nature of their extracellular enzymes, such as lignin Peroxidase (LiP), manganese Peroxidase (MnP), laccase, H₂O₂-generating enzymes, oxidases, etc. (Kirk and Farrell, 1987).

The white rot fungus (WRF) *Phanerochaete chrysosporium* has been widely used as a model system to understand the process of lignin biodegradation. The archetypal WRF *P. chrysosporium* has been shown to degrade a variety of persistent environmental pollutants (Sasek *et al.*, 1993; Barr and Aust, 1994; Vyas *et al.*, 1994a; 1994b; Novotny *et al.*, 1997). There are numerous reports concerning decolorization and degradation of dyes by the enzyme and cultures of *P. chrysosporium* (Glenn and Gold, 1983, Moreira *et al.*, 2000, Novotny *et al.*, 2001, Jarosz-Wilkolazka *et al.*, 2002). In our present work, we have shown that sulphonphthalein (SP) dyes are decolorized by MnP activity produced by shallow stationary nitrogen limited cultures of *P. chrysosporium*.

6.2 Materials and methods

Organism

Phanerochaete chrysosporium strain ME-446 (ATCC 3545) was maintained on malt agar slants and stored at 5 °C.

Production of ligninolytic enzymes by P. chrysosporium

Shallow stationary cultures of *P. chrysosporium* growing on nitrogen-limited mineral medium (Tien and Kirk, 1988) were set up (Vyas *et al.*, 1994c) and incubated at 37 °C. A set of 3 flasks was harvested on 4th day and onwards. Cell free extract was collected after spinning (5600 rpm, 5 °C, 15 min) the content and stored in small aliquots at -5 °C for further biochemical analysis.

Enzyme assays

Lignin peroxidase (LiP) activity was estimated according to Tien and Kirk (1988). One unit of enzyme activity was defined as the amount of activity that will produce one μmol of veratrylaldehyde per min upon oxidation of veratryl alcohol.

Manganese peroxidase (MnP) activity was analysed by spectrophotometric measurement of oxidation of guaiacol to colored product. The reaction mixture (2 ml) contained 100 μmol succinate lactate buffer (pH 4.5), 0.2 μmol MnSO_4 , 0.9 μmol guaiacol, 0.7 μmol H_2O_2 and the enzyme extract. One unit enzyme activity is defined as the amount of activity that produces a change of one A_{470} unit per min upon oxidation of guaiacol.

Sulphonphthalein dye decolorization assays

Sulphonphthalein (SP) dye decolorization by MnP activity was assayed using the same reaction mixture as MnP where in guaiacol was replaced by respective SP dye. The reaction was terminated after 5 min with 1.25 μmol NaOH. Absorbance was monitored at the λ_{max} of respective dyes and dye decolorizing activity was calculated using molar absorbance coefficient of the respective dye

6.3 Results and discussion

Shallow stationary cultures of *Phanerochaete chrysosporium* grown on low nitrogen mineral medium produced manganese peroxidase (MnP) and lignin peroxidase (LP) during secondary metabolism (Fig. 6.1).

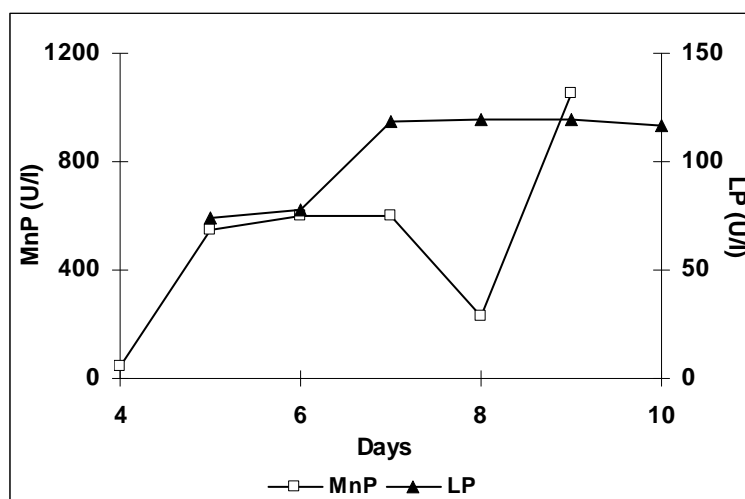


Figure 6.1 Production of lignin peroxidase and manganese peroxidase activities by shallow stationary cultures of *Phanerochaete chrysosporium* growing on nitrogen limiting medium

Manganese peroxidase (MnP; EC 1.11.1.13) was discovered in *P. chrysosporium* (Glenn and Gold 1985; Paszczynski *et al.*, 1985) almost 20 years ago but had been paid less attention earlier compared to LP. MnP oxidizes Mn^{2+} to Mn^{3+} which is stabilized by organic acid chelators and acts in turn as a low molecular mass, diffusible, redox mediator

that attacks organic molecules nonspecifically via hydrogen and one electron abstraction (Wariishi *et al.*, 1988; Gold, 1989).

These enzymes degrade a variety of xenobiotic compounds (Cameron *et al.*, 2000). Decolorization of certain dyes (azo, heterocyclic, polymeric, anthraquinonic, etc.) has been shown to be catalyzed by MnP and LP of *P. chrysosporium* (Jarosz-Wilkolazka *et al.*, 2002). The decolorization of dyes by white rot fungi was first reported by Glenn and Gold (1983), who developed a method to measure ligninolytic activity of *P. chrysosporium* based upon decolorization of a number of polymeric dyes. We report decolorization of several sulphonphthalein (SP) dyes by MnP activity produced by shallow stationary nitrogen limited culture of *P. chrysosporium*.

Sulphonphthalein dyes	λ_{max} (nm)	ϵ (M⁻¹cm⁻¹)	K_m (mM)	V_{max} (U/ml)
Bromocresol green (BCG)	621	39569	13	0.125
Bromocresol purple (BCP)	593	60865	31	0.308
Bromophenol blue (BPB)	583	52663	14	0.065
Bromophenol red (BPR)	564	10735	2000	6.667
Bromophenol blue (BTB)	623	25753	154	0.25
<i>m</i> -Cresol purple (<i>m</i> -CP)	582	32406	42	0.133
<i>o</i> -Cresol red (<i>o</i> -CR)	577	41931	29	0.476
Phenol red (PR)	564	30737	18	0.217
Thymol blue (TB)	600	2786	25	6.667

Table 6.1 λ_{max} , molar absorption coefficients (ϵ) and kinetic constants of various Sulphonphthalein dyes for MnP activity produced by shallow stationary cultures of *Phanerochaete chrysosporium* growing on low nitrogen medium.

Sulphonphthalein (SP) dyes (Table 6.1) were decolorized by MnP activity. Correlation between decolorization of SP dyes and MnP activity levels was observed. There appeared three distinct stages in MnP production and MnP-catalyzed dye decolorization (Fig. 6.1, Fig. 6.2).

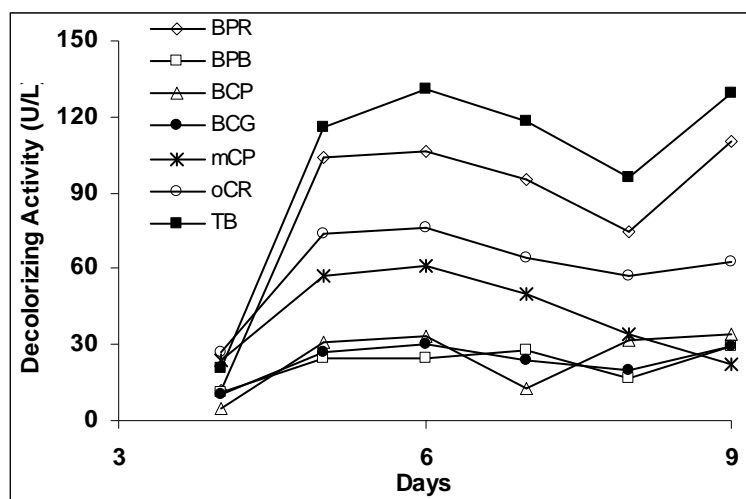


Figure 6.2 Decolorization of BCG, BCP, BPB, BTB, *m*-CP, *o*-CR, PR and TB by manganese peroxidase activity produced by stationary cultures of *Phanerochaete chrysosporium* growing on low-N chemically defined medium

In the first stage there was a rapid development and increase of MnP activity. The second stage was marked by decrease in MnP activity levels. The third stage consisted of rapid increase in MnP activity and reached maximum. MnP decolorizing activity of SP dyes was markedly influenced by pH. Decolorization of all SP dyes occurred optimally at pH 4.0, but that of BCP and BPB was found to occur in a pH range of 3.5 to 4.5 (Fig. 6.3).

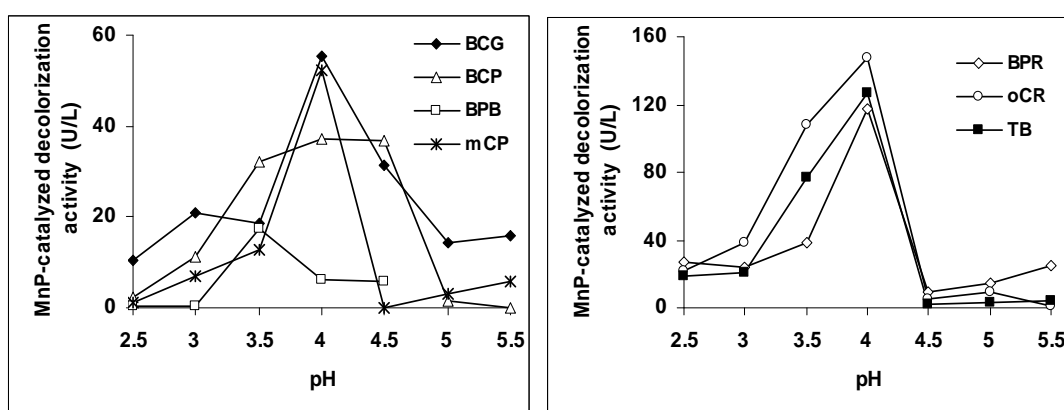


Figure 6.3 Influence of pH on decolorization of BCG, BCP, BPB, BTB, *m*-CP, *o*-CR, PR and TB by manganese peroxidase activity produced by stationary cultures of *Phanerochaete chrysosporium* growing on low-N chemically defined medium

Presence of free phenolic moiety in SP dyes makes them suitable substrates for MnP. This is not surprising as MnP attacks phenolic moieties while degrading lignin. MnP oxidizes various monomeric and dimeric phenols, including phenolic lignin model compounds (Wariishi *et al.*, 1988; 1989). The mechanism is based on initial one electron oxidation of substrate by enzyme-generated Mn^{3+} , which produces phenoxy radical intermediate. This radical is further oxidized by Mn^{3+} to form carbon-centered cation (Tuor *et al.*, 1992).

The method employed for the estimation of SP dyes decolorizing activity involved reaction termination by addition of NaOH. These dyes upon decolorization failed to respond to NaOH addition, indicating loss of pH indicator property. We attribute loss of the color of the dye-substrates and pH indicator property to the degradation of chromophores of dyes.

EDTA, a metal chelator, inhibited MnP-catalyzed decolorization reaction. The inhibitory influence increased with increasing concentration of EDTA in the reaction mixture. Sodium azide, an oxidase inhibitor, completely inhibited MnP decolorization activity. Oxygen scavenger sodium metabisulfite and hydroxyl radical scavengers thiourea and mannitol did not have any influence on MnP decolorization activity. Cysteine, a classical inhibitor of phenol oxidase type activity, retarded the reaction rate in the initial stages and with time the reaction rate gradually picked up (Fig. 6.4).

Inhibition of BCP decolorizing activity of MnP by oxidase inhibitors EDTA, NaN_3 and cysteine is suggestive of oxidative nature of BCP decolorization reaction. Insensitivity of BCP decolorizing activity of MnP to $Na_2S_2O_5$, mannitol and thiourea suggests the non-involvement of molecular oxygen and hydroxyl radicals in MnP catalyzed BCP decolorization.

As compared to BCG and BPB, phenol red had lower K_m and K_s values and higher V_{max} value, indicating that PR is a favored substrate. Similarly *o*-cresol red is a better substrate than BCP (Table 6.1).

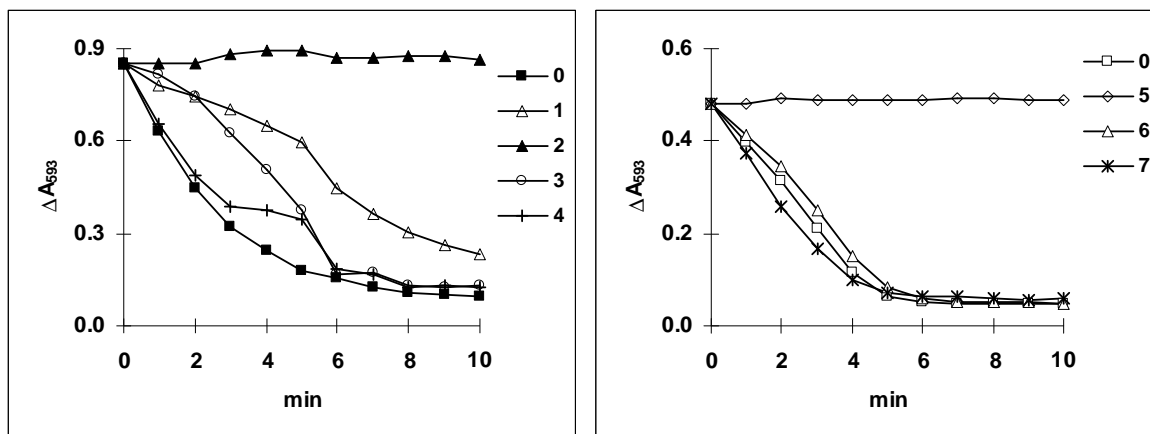
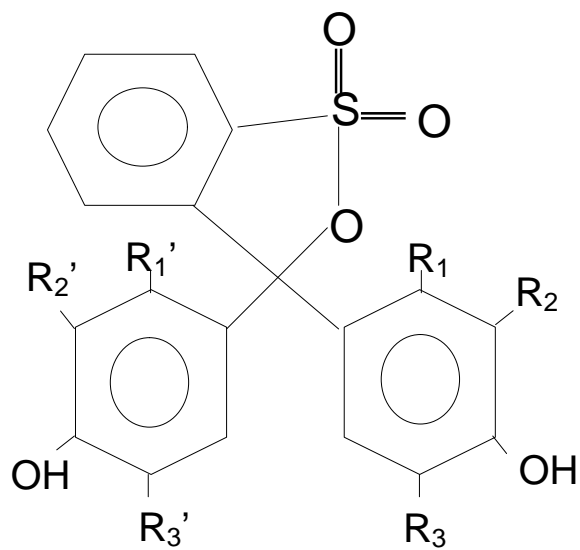


Figure 6.4 Influence of various inhibitors on BCP decolorizing activity of manganese peroxidase produced by stationary cultures of *Phanerochaete chrysosporium* growing on low-N chemically defined medium. [0) control, 1) EDTA (50 μ M), 2) EDTA (250 μ M), 3) Cysteine (75 μ M), 4) Mannitol (250 μ M), 5) Sodium azide (5 μ M), 6) Sodium metabisulfite (250 μ M), 7) Thiourea (500 μ M)].

Presence of additional auxochromes (position, type and number) on the SP dye chromophore influence the suitability of SP dyes as MnP substrate. Methyl group in *ortho* position (*o*-CR) is favored than that in *meta* position (*m*-CP) (Fig. 6.5).

The results demonstrated that SP dyes serve as substrate of MnP and provide another class of chromogen for detection and estimation of ligninolytic peroxidases. Moreover, these dyes that represent priority xenobiotics and recalcitrant compounds are decolorized upon oxidative degradation by MnP, a potent biochemical tool of the white rot basidiomycete *P. chrysosporium*.



Sulphonphthalein dye	$R_1=R_1^1$	$R_2=R_2^1$	$R_3=R_3^1$
Bromocresol green	CH ₃	Br	Br
Bromocresol purple	H	CH ₃	Br
Bromophenol blue	H	Br	Br
Bromophenol red	H	Br	H
Bromothymol blue	CH ₃	Br	CH ₃ -C-CH ₃
<i>m</i> -Cresol purple	CH ₃	H	H
<i>o</i> -Cresol red	H	H	CH ₃
Phenol red	H	H	H
Thymol blue	CH ₃	H	CH ₃ -C-CH ₃

Figure6.5 General structure of sulphonphthalein dyes

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CHAPTER 7

DECOLORIZATION OF SULFONPHTHALEIN DYES BY A LIGNINOLYTIC MANGANESE-INDEPENDENT PEROXIDASE ACTIVITY

7.1 Introduction

Dyes constitute an important family of organopollutants since they are an essential part of our civilization and represent structurally and chemically diverse group of xenobiotic compounds. Synthetic dyes and pigments, released into the environment mainly in the form of wastewater effluents by textile, dyeing, printing, food and leather industries, cause severe ecological problems (Nerud *et al.*, 2001). Physical and chemical methods used for the removal of dyes i.e. adsorption, chemical transformation, incineration, photocatalysis or ozonation, are effective but rather costly (Banat *et al.*, 1996). Dyes being highly resistant to microbial attack are hardly removed from effluents by conventional biological wastewater treatment such as activated sludge (Shaul *et al.*, 1991). There is a need to develop a practical biological method of dye waste treatment that can be used for a wide range of wastes.

The potent biological system involves, white rot fungi (WRF), a group of basidiomycetes characterized by their ability to degrade and mineralize lignin completely in wood (Zabell and Morrell, 1992). Free radical based, unique lignin degrading system of white rot fungi has made them effective over a variety of hazardous environmental pollutants including various dyes (Bumpus *et al.*, 1985; Hammel, 1992; Barr and Aust, 1994). The ability of these fungi to degrade lignin and structurally diverse pollutants results from the relatively nonspecific nature of their extracellular peroxidases (Kirk and Farrell, 1987; Christian *et al.*, 2004).

Differences in the production of such enzymes enable WRF to differ in their ability to degrade and decolorize various dyes. As a result of nonspecificity of lignin-degrading enzymes, individual azo-, heterocyclic-, phthalocyanin-, polymeric-, triphenylmethane-

and anthraquinonic dyes (Ollikka *et al.*, 1993; Swamy and Ramsay, 1999; Novotny *et al.*, 2001; Maximo *et al.*, 2003) as well as complex industrial effluents are efficiently decolorized (Schliephake *et al.*, 1993; Kirby *et al.*, 1995).

Out of many types of synthetic dyes, the Sulfonphthalein (SP) dyes are the least studied structures from the respect of fungal degradation and involvement of peroxidases. SP dyes are pH and redox indicators and tissue stains, resist biodegradation and may persist in the environment. Decolorization of SP dyes even by bacterial system has not yet been reported. Our group recently reported decolorization of SP dyes by manganese peroxidase produced by shallow stationary nitrogen-limited cultures of *Phanerochaete chrysosporium* (Christian *et al.*, 2003).

Pleurotus ostreatus produced manganese-independent peroxidase (MIP) activity along with manganese peroxidase (MnP), phenol oxidase and novel extracellular H₂O₂-requiring RBBR decolorizing enzymatic activities during the solid state fermentation of wheat straw (Vyas and Molitoris, 1995). We have reported here that the SP dyes are decolorized by MIP produced by *P. ostreatus* during solid state fermentation.

7.2 Materials and methods

Organism and culture conditions

Pleurotus ostreatus 3004, a strain of cultivated mushroom was maintained on malt agar slants at 5 °C. Transfers were made on malt agar plates and the strain was cultivated at room temperature. Five g of neatly chopped wheat straw was taken in 250-ml Erlenmeyer flasks, moistened with 20 ml distilled water, autoclaved (121 °C, 20 min x 3 cycles) and used for solid state fermentation. Ten agar disks punched from the malt agar plates fully covered with mycelia were used to inoculate the straw. The flasks were kept at room temp in dark and harvested when entire content of the flasks was covered with mycelial growth.

Production and preparation of extracellular enzyme extract

A set of five flasks was harvested and processed for the preparation of extracellular enzyme extract according to Vyas *et al.*, (1994). Total protein in the enzyme extract was precipitated with ammonium sulphate (80% saturation), separated by vacuum filtration,

redissolved in phosphate buffer and spun (5000 rpm, 15 min, 5 °C). The clear supernatant obtained upon centrifugation was used as enzyme sample for various biochemical studies.

Biochemical analyses

Decolorization of sulfonphthalein (SP) dyes was monitored at the absorbance maxima of the respective dyes. The reaction mixture for manganese-independent peroxidase (MIP) contained in 2 ml, 100 µmol tartarate or succinate lactate buffer (pH 3.5 or 4.0), 0.05-0.5 µmol dye, 0.2 µmol H₂O₂ and enzyme sample. Reactions were initiated by the addition of H₂O₂ and terminated with 500 µmol NaOH after 10 min incubation period. All the reactions were carried out at room temperature. Dye decolorizing activity was calculated using molar absorption coefficient of the respective dye. One unit enzyme activity is the amount of enzyme that consumes 1 µmol of dye per min in the reaction mixture.

7.3 Results and discussion

White rot fungi (WRF) produce several extracellular oxidases and peroxidases involved in the degradation of lignocellulosic substrates (Kirk and Farrell, 1987; Christian *et al.*, 2004). Fungal lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase have been implicated in the decolorization of diverse synthetic dyes (Ollikka *et al.*, 1993; Chivukula and Renganathan, 1995; Heinfling *et al.*, 1998; Schliephake *et al.*, 2000; Moreira *et al.*, 2001).

Pleurotus ostreatus produces different dye decolorizing activities along with the production of ligninolytic enzymes (Vyas and Molitoris, 1995; Novotny *et al.*, 2001). Decolorization of sulfonphthalein (SP) dyes namely phenol red (PR), bromophenol red (BPR) and bromophenol blue (BPB) by the extracellular enzyme preparation was observed to be influenced by Mn²⁺ and pH. Enzymatic decolorization of PR and BPR was positively influenced by the increasing concentration of Mn²⁺ at pH 4.0 (Fig. 7.1a, c). Increases in Mn²⁺ conc (upto 15 µM) resulted in increased inhibition of BPB decolorization. Further increase in Mn²⁺ conc did not cause corresponding increase in inhibition, exhibiting saturation kinetics (Fig. 7.1b).

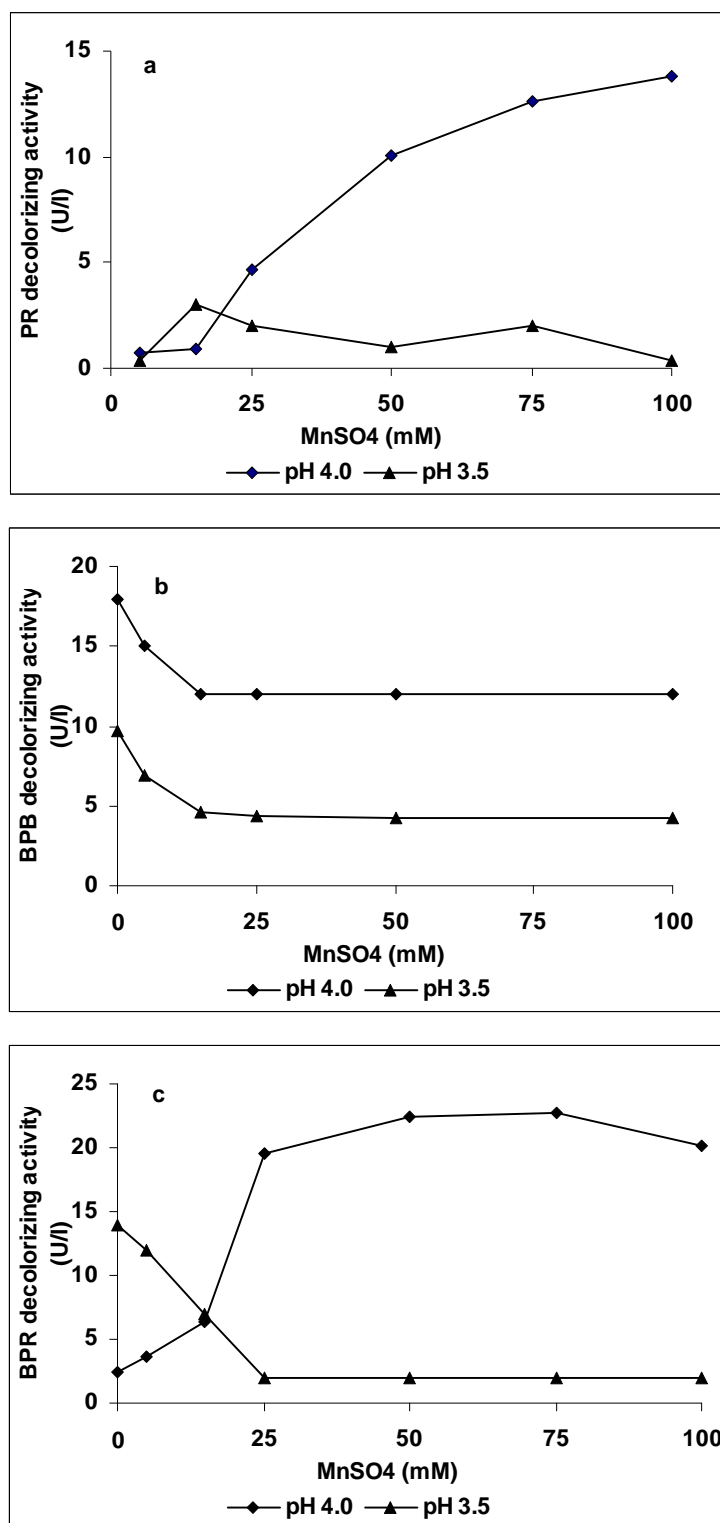
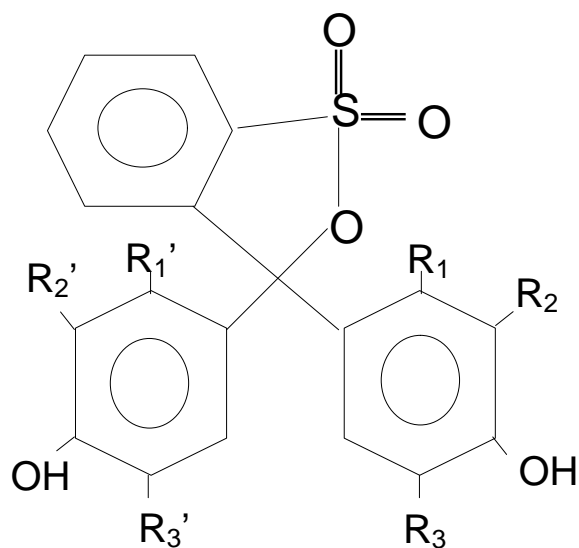


Figure 7.1 Influence of Mn^{2+} at pH 4.0 () and 3.5 () on phenol red (a), bromophenol blue (b) and bromophenol red (c) decolorizing enzymatic activity produced by *Pleurotus ostreatus* during solid-state fermentation of wheat straw

Decolorization of SP dyes in the absence of Mn^{2+} by enzyme preparation and production of manganese-independent peroxidase (MIP) activity by *P. ostreatus* led us to assess the role of MIP in BPB decolorization. MnP of *Bjerkandera adusta* and *Pleurotus eryngii* decolorized azo and heterocyclic dyes in a Mn^{2+} -independent manner (Ollikka *et al.*, 1993; Heinfling *et al.*, 1998).

At pH 3.5 BPB and BPR decolorization occurred in the absence of Mn^{2+} . Mn^{2+} (5 μM) in reaction mixture inhibited 14% of BPR decolorization whereas same concentration inhibited 30% BPB decolorization, which increased further upon subsequent increase in the concentration of Mn^{2+} (Fig. 7.1b, c). At this pH, Mn^{2+} showed neither stimulatory nor inhibitory influence on PR decolorization (Fig. 7.1a).

Presence of additional auxochromes i.e. bromine at 2, 2', 3 and 3' positions on BPB chromophore influences the suitability of BPB as MnP substrate (Fig. 7.2).



Sulphonphthalein dye	$R_1=R_1'$	$R_2=R_2'$	$R_3=R_3'$
Phenol red	H	H	H
Bromophenol red	H	Br	H
Bromophenol blue	H	Br	Br

Figure 7.2 General structure of sulphonphthalein dyes

Electronegativity of bromine atom makes the hydroxyl group on the chromophore electron deficient, thereby making BPB a poor substrate of MnP. Lower K_m for BPB and higher V_{max} in MIP-catalyzed BPB decolorization reaction indicated that the BPB is a favoured substrate of MIP at pH 3.5 (Table 7.1). Decolorizing activity of MIP was generally observed to be higher at pH 3.5 than at pH 4.0. It did not decolorize PR at either pH, decolorized BPR better at 3.5 than at 4.0. BPB was decolorized rapidly by MIP at both the pH (Fig. 7.1).

SP Dye	MnP		MIP	
	K_m (μ M)	V_{max} (U/l)	K_m (μ M)	V_{max} (U/l)
Phenol red	40	9.4	-	-
Bromophenol red	60	48	-	-
Bromophenol blue	-	-	38	9.4

Table 7.1 Kinetic constants for sulfonphthalein dyes of manganese peroxidase and manganese-independent peroxidase

The order of substrate preference shown by MIP is BPB > BPR > PR. PR did not serve as a substrate. Halogenation makes the dye compound a better substrate of MIP but poorer substrate of MnP. This proposes the possible exploitation of MIP for the degradation of halogenated aromatic compounds with higher degree of halogenation.

A novel ligninolytic peroxidase, produced by *Pleurotus* and *Bjerkendera* sp. described as versatile peroxidase (VP), combines the catalytic properties of MnP and LiP. VP is able to carryout oxidation of both veratryl alcohol and Mn^{2+} to veratryl aldehyde and Mn^{3+} respectively (Camarero *et al.*, 1999). *P. ostreatus* did not produce LiP and veratryl alcohol oxidase during SSF of wheat straw. MIP-catalyzed BPB decolorizing activity (i) is inhibited by Mn^{2+} , (ii) does not oxidize Mn^{2+} and (iii) does not oxidize veratryl alcohol. This distinguishes MIP activity from MnP, VP and lignin peroxidase.

BPB decolorization by MIP activity was observed to be H_2O_2 -dependent. H_2O_2 concentration higher than optimal inhibited decolorizing activity (Fig. 7.3).

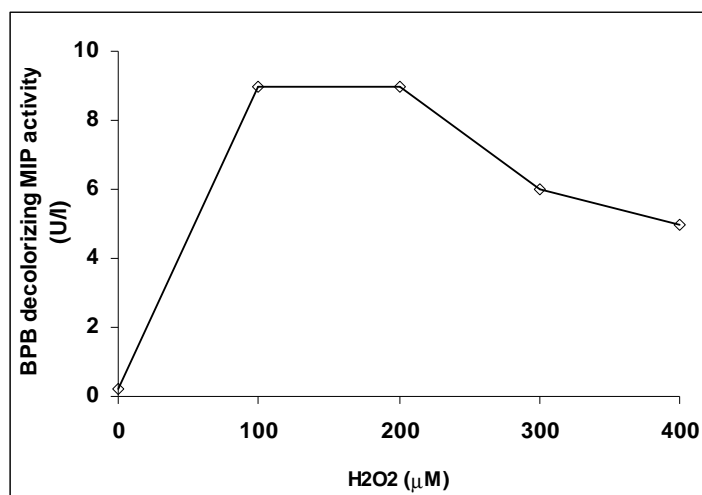


Figure 7.3 Influence of H₂O₂ on bromophenol blue decolorizing manganese-independent peroxidase activity produced by *Pleurotus ostreatus* during solid-state fermentation of wheat straw

The influence of various inhibitors on BPB decolorization by MIP activity was investigated. EDTA is a metal chelator and acts on catalytic metal centre. The inhibitory influence increased with increasing concentration of EDTA in the reaction mixture. Dependence on H₂O₂, inhibition by higher than optimal concentration of H₂O₂ and inhibition by EDTA of BPB decolorization suggests the presence and involvement of catalytic metal centre in BPB decolorization by this enzymatic activity (Fig. 7.4).

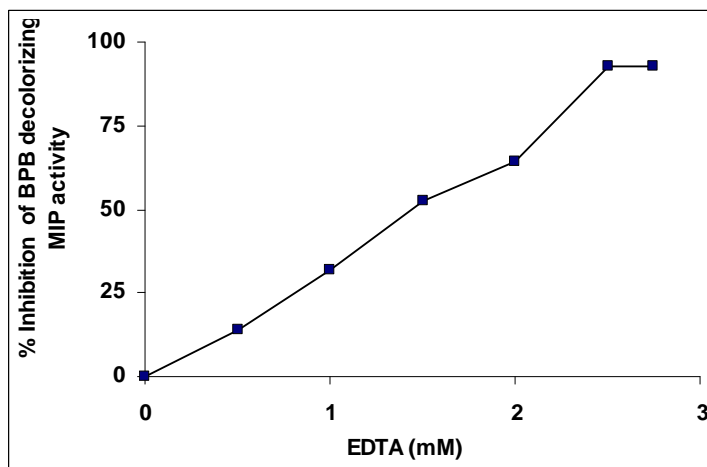


Figure 7.4 Inhibition by EDTA of bromophenol blue decolorization by manganese-independent peroxidase produced by *Pleurotus ostreatus* produced during solid-state fermentation of wheat straw

Sodium azide, a strong oxidase inhibitor, at 2.5 μM concentration in the reaction mixture, inhibited 54% of BPB decolorization. 10 μM sodium azide caused 90% inhibition (Fig. 7.5).

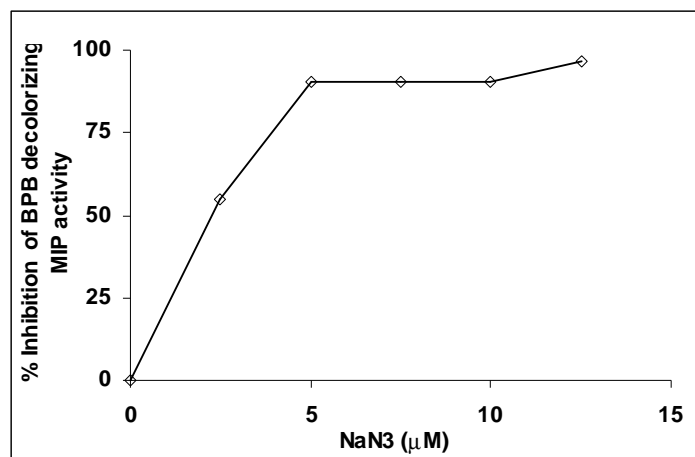


Figure 7.5 Inhibition by sodium azide of bromophenol blue decolorization by manganese-independent peroxidase produced by *Pleurotus ostreatus* produced during solid-state fermentation of wheat straw

Sodium metabisulfite, molecular oxygen scavenger, inhibited BPB decolorization by 32% and 50% respectively at 100 and 400 μM conc. (Fig. 6a). It inhibited BPB decolorization non-competitively indicated by decreasing V_{max} and constant K_m (Fig. 6b). Inhibition of BPB decolorization reaction by metabisulfite implies that it is an oxygenation reaction and occurs in the presence of molecular oxygen. MIP-catalyzed decolorization of BPB is an oxidative process.

The non-specific nature of oxidation of lignin by white rot fungi suggests that extracellular reactive oxygen species have a role in this process. Hydroxyl radical is a reasonable candidate for wood decay agent because it is the strongest oxidant that can occur in aqueous system (Hammel *et al.*, 2002). It reacts rapidly with virtually all organic molecules, either by abstracting hydrogens from aliphatic structures or by adding as an electrophile to aromatic ones (Halliwell and Gitteridge, 1999). Furthermore, hydroxyl radical attacks the subunit of lignin both by abstracting aliphatic C -hydrogens and by adding to aromatic rings (Gierer, 1990). LiP, produced by ligninolytic cultures of

Phanerochaete chrysosporium, generates hydroxyl radicals (Barr *et al.*, 1992). Kerem *et al.*, (1992) suggested that during ligninolysis by *P. ostreatus* that lacks lignin peroxidase (LP) activity, enzymatic activity other than those of MnP and LiP is involved in the production of free radicals by this fungus.

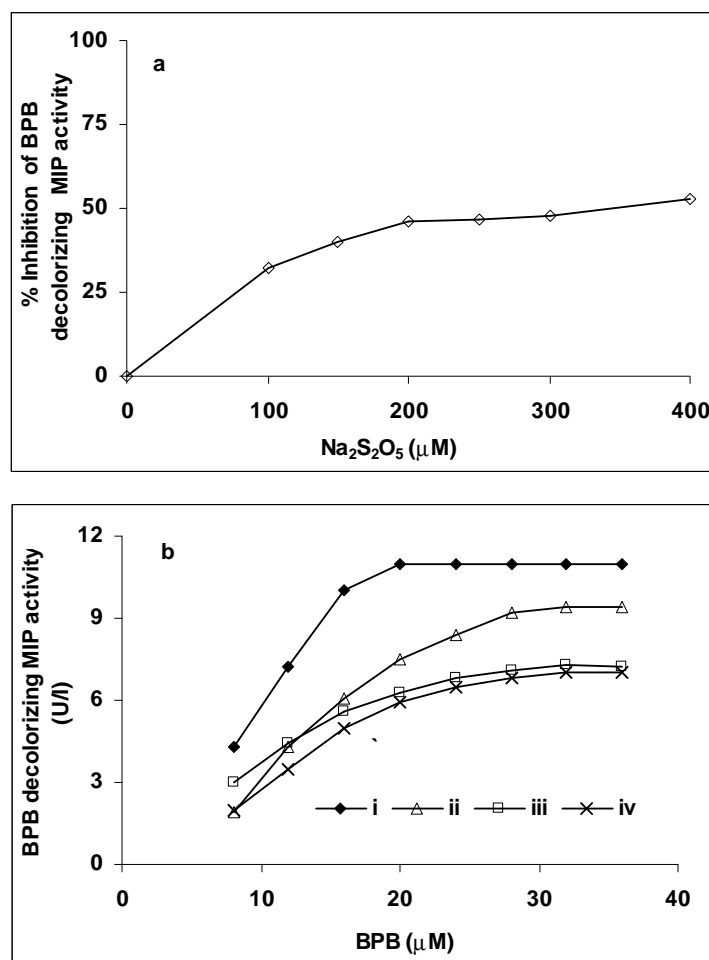


Figure 7.5 (a) Inhibition by varying concentration sodium metabisulfite on manganese-independent peroxidase catalyzed decolorization of bromophenol blue (25 μM)
 (b) Inhibition by varying concentration of sodium metabisulfite [0 μM (i), 100 μM (ii), 150 μM (iii) and 250 μM (iv)] of manganese-independent peroxidase catalyzed decolorization of bromophenol blue at varying concentration

Thiourea, a hydroxyl radical scavenger, inhibited MIP-catalyzed BPB decolorization competitively evidenced by the fact that increasing concentration of thiourea resulted in increase in the K_m value for BPB without any change in V_{max} (Table 7.2). As the concentration was increased from 50 μM to 250 μM , BPB was found to be inhibited further from 41% to 87% (Fig. 7.7). These results are suggestive of generation of hydroxyl radicals by MIP and participation of hydroxyl radicals in the decolorization of BPB.

Thiourea (μM)	K_m (μM)	V_{max} (U/ml)
0	33.3	0.025
25	70	0.025
50	100	0.025
100	500	0.025

Table 7.2 Kinetic constants for bromophenol blue in the presence of thiourea of bromophenol blue decolorizing manganese-independent peroxidase activity

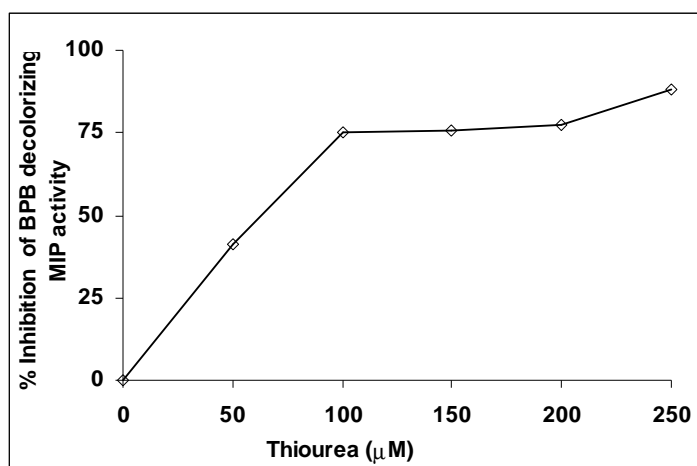


Figure 7.7 Inhibition by thiourea of bromophenol blue decolorization by manganese-independent peroxidase produced by *Pleurotus ostreatus* produced during solid-state fermentation of wheat straw

Decolorization of SP dyes by MIP is an oxidative process involving oxygenation and also hydroxyl radicals. The MIP produced by white rot fungus *P. ostreatus* appears to be an important tool for the decolorization of synthetic dyes and also degradation of halogenated compounds. Isolation of MIP and its role in the generation of hydroxyl radical, dehalogenation and degradation of xenobiotic pollutants and lignin is being investigated.

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CHAPTER 8

DECOLORIZATION OF TEXTILE DYES BY FENTON REAGENT

8.1 Introduction

A great variety of synthetic dyes are released into the environment in effluents arising from major processes in the textile dyeing and other industrial applications. The structural diversity of dyes is derived from the use of different chromophoric groups and different application technologies. Between 10-15% of total dye consumed in dyeing processes may be found in wastewater. Dyes are usually aromatic and heterocyclic compounds and are recalcitrant, some of them being toxic and even carcinogenic. Most of these compounds are highly resistant to microbial attack and therefore, it is hard to remove them from effluents by means of conventional biological treatment such as activated sludge. In this regard, efforts to develop new strategies are needed for the removal of these contaminants.

H. J. H. Fenton discovered the strong oxidizing power of mixture of hydrogen peroxide and Fe(II) that is known as Fenton reagent for more than hundred years ago (Fenton, 1894). Hydrogen peroxide, when catalyzed by ferrous ions, generates a strong nonspecific oxidant hydroxyl radical that reacts with most organic compounds at high diffusion rates (Dorfman and Adams, 1973).

Use of Fenton chemistry for the decolorization of dyes has not been widely studied. A recent study however, reported decolorization of synthetic dyes by Fenton system (Nerud *et al.*, 2001). The present study was conducted to test the efficacy of Fenton reagent for the decolorization of textile dyes.

8.2 Materials and methods

Chemicals

Seven different textile dyes Remazol magenta HB (A₅₅₇), Reactive blue 21 (A₆₂₀), Remazol red H8B (A₅₀₉), Reactive orange 13 (A₄₈₀), Reactive brown 18 (A₄₅₂), Reactive

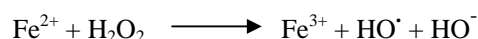
black 5 (A₅₉₆) and Remazol brilliant blue R (A₅₉₅) were used for decolorization study. Fe(II) as FeSO₄ (1 mM) and hydrogen peroxide (10 mM) were added as aqueous solutions to the reaction mixture to give final concentrations as indicated in parenthesis.

Decolorization of dyes

Textile dyes (100 ppm) were added to the reaction mixture as aqueous solutions. The dyes were incubated with Fenton reagent and decolorization was measured at the absorbance maxima of the respective dyes for half an hour using Shimadzu UV-1601 spectrophotometer. The decolorization was initiated by the addition of hydrogen peroxide to the reaction mixture. The incubation proceeded at room temperature in the dark. The reaction mixture was also scanned (240-800 nm) before and after decolorization. Thiourea (10 mM) was used as hydroxyl radical scavenger.

8.3 Results and discussion

Although many wood decay basidiomycetes secrete oxidative and hydrolytic enzymes that participate in lignocellulose biodegradation, it is generally recognized now that these enzymes cannot penetrate sound wood, and that fungi must employ smaller agents to initiate decay. Reactive oxygen species are likely candidates, and evidence is accumulating that wood decay fungi produce these oxidants. Hydroxyl radical has been implicated in all the three major types of wood decay caused by fungi, viz. white rot, brown rot and soft rot (Backa *et al.*, 1992; 1993; Tanaka *et al.*, 2000). Hydroxyl radical has received more attention than other reactive oxygen species in studies of wood decay, in part because there is a well-recognized route for its production in biological systems via the Fenton reaction. Fenton reagent is produced by different routes in wood degrading fungi (Wood, 1994; Hammel *et al.*, 2002).



We used a similar system to investigate the Fenton reaction mediated-decolorization of textile dyes. Decrease in the absorbance at λ_{max} of respective dye in the presence of Fenton reagent was a function of time. Fenton reaction efficiently decolorized remazol magenta HB, remazol read H8B and reactive orange 13. Reactive black 5 and remazol brilliant blue R were decolorized completely (Table 8.1).

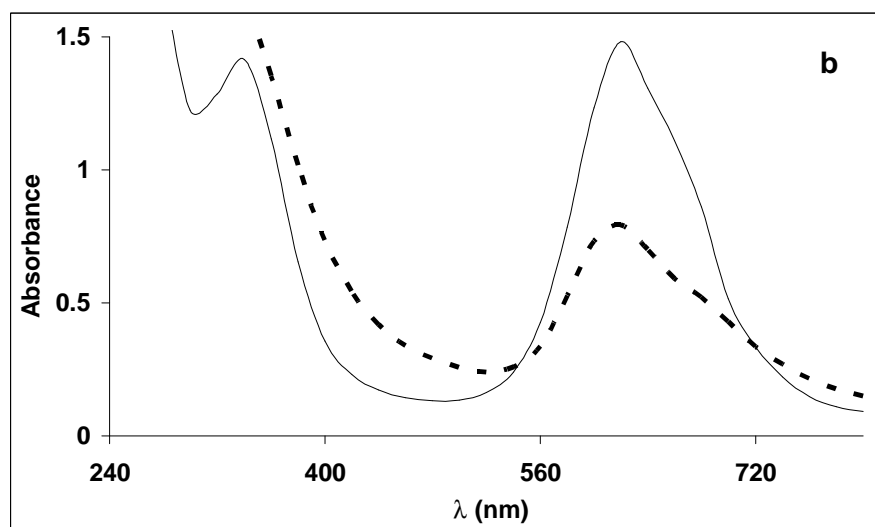
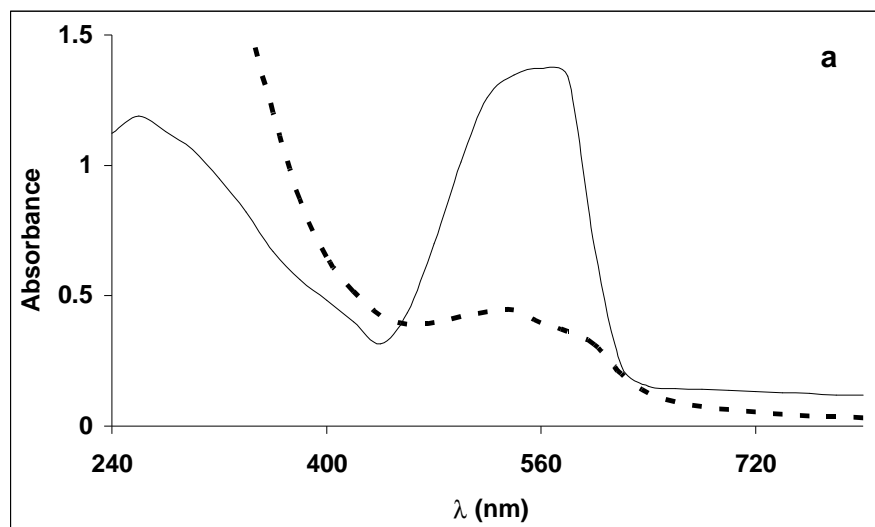
Dyes	% Decolorization
Remazol magent HB	69.6
Reactive blue 21	44.8
Remazol red H8B	85.5
Reactive orange 13	74.9
Reactive brown 18	51.7
Reactive black 5	90.6
Remazol brilliant blue R	92.9

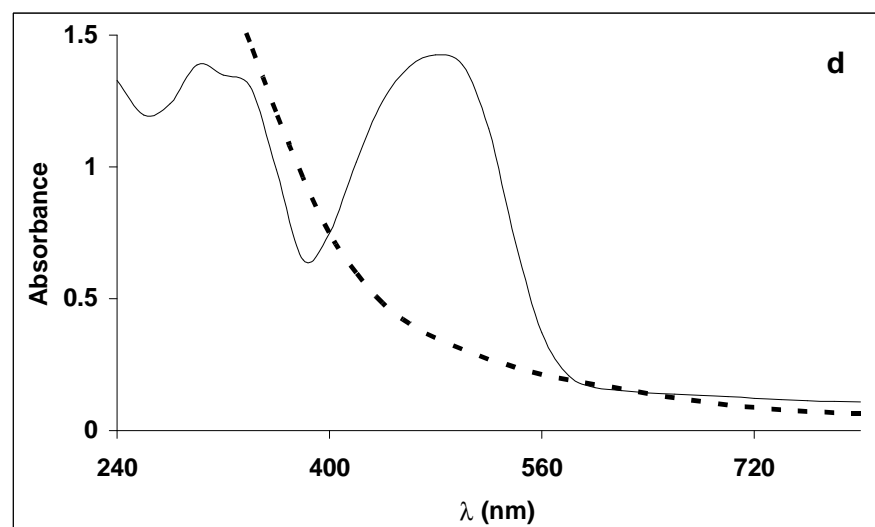
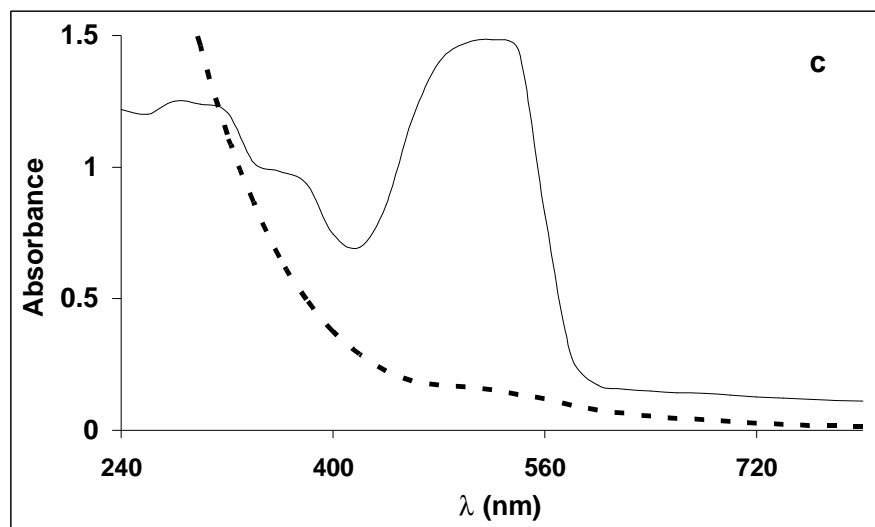
Table 8.1 Decolorization of seven textile dyes by Fenton reagent.

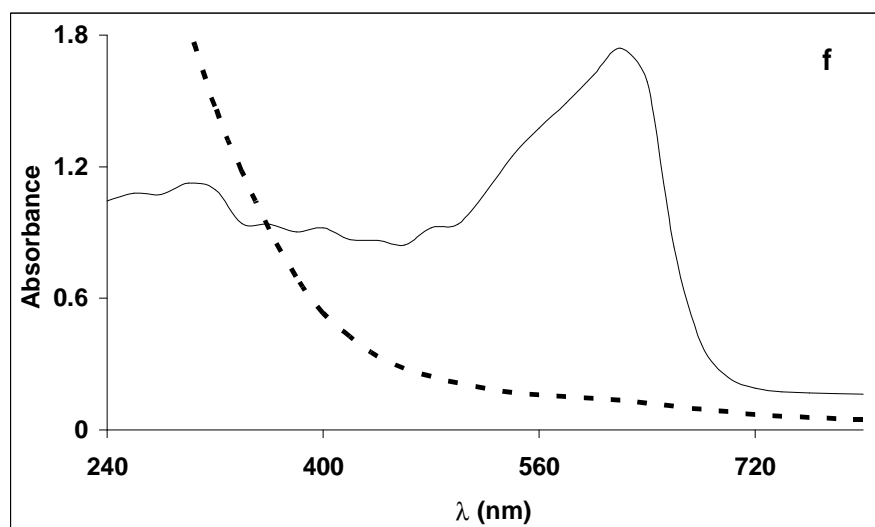
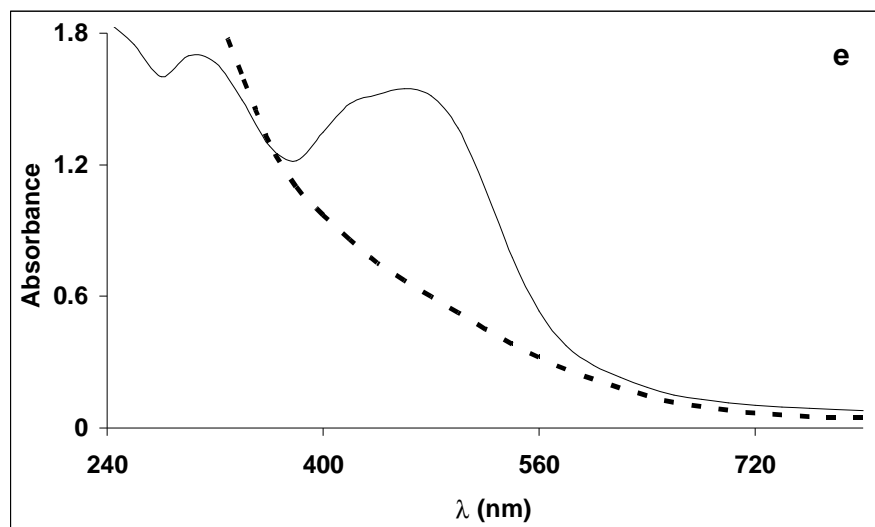
The decolorization reaction followed characteristic sequential changes in the color from blue to colorless through a series of intermediates also observed earlier with the enzyme-catalyzed dye decolorization reactions (Vyas and Molitoris, 1995), suggesting decolorization is not a single step reaction and that various intermediates are formed during the decolorization process. Substantial decolorization of the dyes was observed immediately after initiating the reaction with hydrogen peroxide. Reactive blue 21 and reactive brown 18 were decolorized partially within 30 min. These dyes also resist decolorization by white rot fungi growing on solid media (Unpublished data).

Upon decolorization by Fenton reagent absorbance maxima of the dyes tested shifted towards lower wavelength (Fig. 8.1). Similar results were obtained with decolorization by modified Fenton reagent using Cu / pyridine / hydrogen peroxide (Nerud et al., 2001). Such hypsochromic shift is suggestive of degradation of the molecule through a series of intermediates (Gold *et al.*, 1988; Vyas and Molitoris, 1995).

White rot and brown rot fungi produce hydroxyl radical at a distance from the fungal hyphae, ideally within the lignified secondary cell wall during lignocellulose degradation. Hydroxyl radical is a reasonable candidate for wood decay during lignin degradation because it is the strongest oxidant that can occur in aqueous system. It reacts rapidly with virtually all organic molecules, either by abstracting hydrogens from aliphatic structures or by adding as an electrophile to aromatic ones (Halliwell and Gutteridge, 1989).







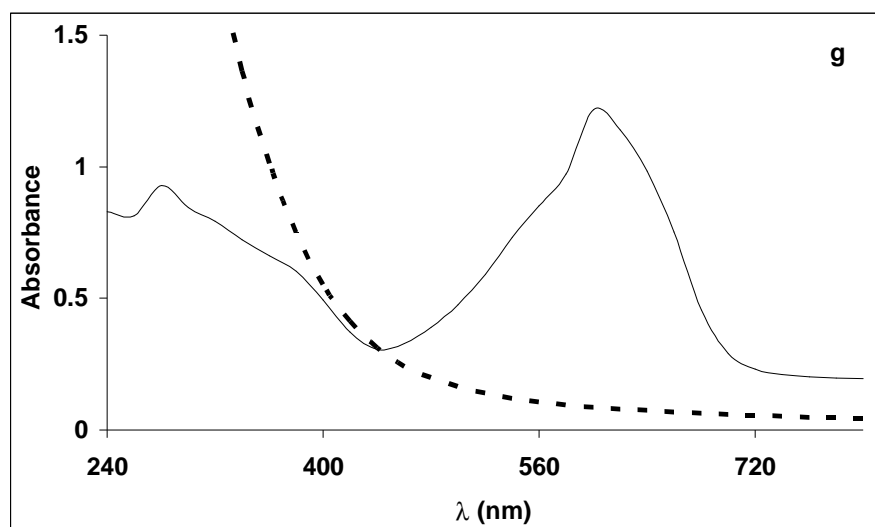


Figure 8.1 UV-visible spectra of dyes before (solid line) and after 30 min decolorization with Fenton reagent (dashed line): (a) Remazol magenta HB; (b) Reactive blue 21; (c) Remazol red H8B; (d) Reactive orange 13; (e), Reactive brown 18; (f) Reactive black 5; (g) Remazol brilliant blue R.

Addition of thiourea (10 mM), a classical hydroxyl radical scavenger, to the Fenton reagent completely inhibited decolorization reaction, provided strong evidence of the involvement of hydroxyl radical in Fenton reagent-catalyzed decolorization reaction.

The results provide an evolution of new non-enzymatic biological system for dealing with problematic pollutants in wastewaters.

8.4 References

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HIGHLIGHTS OF THE STUDY

The purpose of the study was to investigate the ligninolytic system of lignin degrading basidiomycetes and evaluate its potential to study degradation of xenobiotic compounds. Decolorization of dyes was used as a measure to study the potential of lignin-degrading fungi for degrading variety of structurally diverse xenobiotic compounds. The dye decolorization studies are being used as a possible, easily usable and inexpensive alternative to radiolabelled lignins and other xenobiotics in evaluating ligninolytic system and biodegradation studies.

🔗 *Irpex lacteus*, *Agrocybe cylindracea* and *Pleurotus ostreatus vampola* were screened for their ability to decolorize textile dyes on malt agar medium. *Irpex lacteus* that efficiently decolorized all textile dyes was selected for further study. Reactive blue 21, reactive black 5 and remazol brilliant blue R dyes were completely decolorized by day 12. Remazol magenta HB was only partially decolorized upto day 12 and extensively decolorized by day 18 leading to complete loss of the color in the medium. *Irpex lacteus* decolorized reactive blue 21 gradually along the experimental run and was completely decolorized in contrast to its decolorization on solid media. Submerged cultures of *I. lacteus* decolorized effluents E-1, E-2 and E-3 extensively within 18 days. *Irpex lacteus* produced manganese peroxidase (MnP), manganese-independent peroxidase (MIP), lignin peroxidase (LiP) and laccase as early as day 3. *Irpex lacteus* extensively decolorized different textile dyes and also complex textile effluents. The correlation between appearance of ligninolytic activities in the culture filtrate and initiation of decolorization can be attributed to the involvement of ligninolytic activities in the decolorization.

🔗 Most information on biodegradation of synthetic dyes by WRF has been obtained with *Phanerochaete chrysosporium* and few other species. Production of ligninolytic enzymes by *I. lacteus* during solid-state fermentation of natural lignocellulose substrate, wheat straw, and decolorization of sulfonphthalein dyes by ligninolytic enzymes was studied. *Irpex lacteus* under identical conditions produced manganese peroxidase (MnP), manganese-independent peroxidase (MIP), lignin peroxidase and laccase. MnP decolorizes *o*-cresol red, phenol red, bromocresol green and *m*-cresol purple but not

bromophenol blue. On the contrary additional Br groups improved the oxidizability of bromophenol blue by MIP. MIP activity is different from versatile peroxidase and lignin peroxidase since MIP-catalyzed dye decolorization reactions are inhibited in the presence of Mn^{2+} . Sulfonphthalein dye decolorization by MnP and MIP is an oxidative process requiring molecular oxygen and the presence and involvement of catalytic metal centre of the enzymes in the decolorization reaction. Upon decolorization all sulfonphthalein dyes loose the pH indicator property attributing extensive degradation of dye chromophore by MnP and MIP activities. *Irpex lacteus* appears to be a potent organism for decolorization of dyes and degradation of other organopollutants as dye decolorizing ability is used as an indicator for screening the efficient organisms for pollutants degradation.

Ⓒ Paucity of information about the xenobiotic and lignin degrading abilities of litter basidiomycetes prompted us to undertake the isolation and identification of litter basidiomycetes. *Geastrum triplex* is one of the several litter basidiomycetes strains isolated in our laboratory. The litter basidiomycete *G. triplex* was found to produce ligninolytic activities manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase activities under nutrient limiting conditions. The litter basidiomycete *G. triplex* possesses the ability to decolorize structurally diverse dyes. The MnP activity of *G. triplex* decolorized phenol red, reactive blue H5G, remazol magenta HB and fast green but not bromophenol blue. Decolorization of triphenylmethane dyes methyl violet and fast green by MnP was poor. LiP activity decolorized sulfonphthalein, triphenylmethane and textile dyes. Reactive blue, remazol magenta and bromophenol blue were more favorable for decolorization by LiP activities. Differences in the decolorization profiles imply that the isozyme composition representing MnP and LiP activities changes with time.

Ⓒ Lignin peroxidase produced by *Trametes versicolor* decolorizes Remazol brilliant blue R (RBBR) in the presence as well as in the absence of veratryl alcohol (VA). VA enhances and stabilizes the RBBR-decolorization rates by lignin peroxidase. Improvement of RBBR decolorization rates in the presence of VA suggests that VA serves as a mediator in the LiP-catalyzed RBBR decolorization. Mediator role of VA in RBBR decolorization by following simultaneously VA oxidation and RBBR decolorization by LiP was evaluated. It was observed that increases in RBBR concentration increased the decolorization rates. RBBR compete with VA and is the preferred substrate, giving a lag period preceding veratryl aldehyde formation.

④ *Phanerochaete chrysosporium* has been widely used as a model system to understand the process of lignin biodegradation. The fungus was shown to degrade a variety of persistent environmental pollutants. Shallow stationary cultures of *P. chrysosporium* grown on N-limited medium produced MnP and LiP during secondary metabolism. Decolorization of several sulphonphthalein (SP) dyes by MnP produced by shallow stationary nitrogen limited culture of *P. chrysosporium* was studied. Presence of free phenolic moiety in SP dyes makes them suitable substrates for MnP. The results demonstrated that SP dyes serve as substrate of MnP and provide another class of chromogen for detection and estimation of ligninolytic peroxidases. Moreover, these dyes that represent priority xenobiotic and recalcitrant compounds are decolorized upon oxidative degradation by MnP, a potent biochemical tool of the white rot basidiomycete *P. chrysosporium*.

④ White rot fungus *Pleurotus ostreatus* produced manganese-independent peroxidase (MIP) along with manganese peroxidase, laccase and RBBR oxygenase during solid-state fermentation of wheat straw. Decolorization of SP dyes in the absence of Mn^{2+} by enzyme preparation and production of manganese-independent peroxidase (MIP) activity by *P. ostreatus* prompted to assess the role of MIP in BPB decolorization. Presence of additional auxochromes i.e. bromine at 2, 2', 3 and 3' positions on BPB chromophore influences the suitability of BPB as MnP substrate. Electronegativity of bromine atom makes the hydroxyl group on the chromophore electron deficient, thereby making BPB a poor substrate of MnP. Decolorization of SP dyes by MIP is an oxidative process involving oxygenation and also hydroxyl radicals. The MIP produced by white rot fungus *P. ostreatus* appears to be an important tool for the decolorization of synthetic dyes and also degradation of halogenated compounds.

④ A non-enzymatic biological system Fenton reagent produced by the ligninolytic fungi during the wood decay was found potential for decolorization of textile dyes. Seven different textile dyes Remazol magenta HB (A₅₅₇), Reactive blue 21, Remazol red H8B, Reactive orange 13, Reactive brown 18, Reactive black 5 and Remazol brilliant blue R were used for decolorization study. Fenton reaction efficiently decolorized remazol magenta HB, remazol read H8B and reactive orange 13. Reactive black 5 and remazol brilliant blue R were decolorized completely. Reactive blue 21 and reactive brown 18 were decolorized partially within 30 min.

APPENDIX 1

SCHOLARSHIPS AWARDED

- ④ Senior Research Fellowship from Council of Scientific and Industrial Research (CSIR), New Delhi, India (May, 2003 till date).
- ④ Junior Research Fellowship from Department of Special Assistance (DSA), India (January, 2002-April, 2003).

APPENDIX 2

LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1. **Christian, V.V.**, Shrivastava, R., Novotny, C., Vyas, B. R. M.: Decolorization of sulfonphthalein by manganese peroxidase activity of white rot fungus *Phanerochaete chrysosporium*. *Folia Microbiol.* **48** (In press) (2003)
2. **Christian, V.**, Shrivastava, R., Modi, H. A., Vyas, B.R.M.: Physiology and biochemistry of lignin degradation by white-rot fungi. (Review article) *J. Tissue Res.* **4**, 165-178 (2004).
3. **Christian, V.**, Shrivastava, R., Modi, H. A., Vyas, B. R. M.: Decolorization of sulfonphthalein dyes by a ligninolytic manganese-independent peroxidase activity (Communicated).
4. **Christian, V.**, Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B.R.M.: Sulfonphthalein dye decolorization by ligninolytic enzymes of *Irpex lacteus* produced during solid-state fermentation (Communicated).
5. **Christian, V.**, Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B.R.M.: Ligninolytic enzymes production and decolorization of synthetic dyes by litter basidiomycete *Geastrum triplex* (Communicated).
6. **Christian, V.**, Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B.R.M.: Decolorization of textile dyes and dye industry effluents by *Irpex lacteus* (Communicated).
7. Shrivastava, R., **Christian, V.**, Vyas, B. R. M.: Enzymatic decolorization of sulfonphthalein dyes. (Communicated)

8. **Christian, V.**, Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B.R.M.: Decolorization of textile dyes by Fenton reagent (Communicated).
9. **Christian, V.**, Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B.R.M.: Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: Enzymology and mechanisms involved (Review article) (Communicated).

PRESENTATIONS

1. **Christian V.V.**, Shrivastava R., Ladhawala K.P. and Vyas B.R.M. Decolorization of Sulphonphthalein dyes by manganese peroxidase from lignin degrading basidiomycetes *Phanerochaete chrysosporium*. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India organized by Agharkar Research Institute, Pune. Maharashtra. 6-7 February 2003.
2. Shrivastava R., **Christian V.V.**, Ladhawala K.P. and Vyas B.R.M. Influence of Mn^{II} and NaN₃ on dye decolorization capabilities of peroxidases from *Pleurotus ostreatus*. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India organized by Agharkar Research Institute, Pune. Maharashtra. 6-7 February 2003.
3. Kesharia M. H., **Christian V. V.**, Shrivastava R. and Vyas B.R.M. Dye decolorization capabilities of peroxidases from Litter Basidiomycetes. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India organized by Agharkar Research Institute, Pune. Maharashtra. 6-7 February 2003.
4. Dave M. N., Shrivastava R., **Christian V.V.**, and Vyas B.R.M. Decolorization of textile dyes by the ligninolytic enzymes of white rot basidiomycetes. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India organized by Agharkar Research Institute, Pune. Maharashtra. 6-7 February 2003.

5. Parekh J. T., Shrivastava R., **Christian V.V.**, and Vyas B.R.M. Ligninolytic enzymes of white rot basidiomycetes: Decolorization of textile dyes. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India organized by Agharkar Research Institute, Pune. Maharashtra. 6-7 February 2003.
6. Shrivastava R., **Christian V.V.**, and Vyas B.R.M. Production profile of peroxidases and dye decolorization capabilities of white rot basidiomycetes. National Conference on Environmental Biology, Department of Biosciences, Saurashtra University, Rajkot, Gujarat. October 17-18, 2002.
7. **Christian V.V.**, Shrivastava R., Avlani V.A., and Vyas B.R.M. Screening of ligninolytic enzymes of selected white rot fungi in the decolorization of dye. National Conference on Fungal Diversity and Biotechnology organized by Mycological Society of India, Thane, Maharashtra. February 2-4, 2002

APPENDIX 3

CONFERENCES / SEMINARS / WORKSHOPS ATTENDED

1. Workshop on **Recombinant DNA Technology** organized by Central Facility for Biotechnology Teaching and Research (CFBTR), Madurai, Tamilnadu. September 27- October 5, 2003.
2. National Symposium on **Prospecting of Fungal Diversity and Emerging Technologies & 29th Annual meeting of Mycological Society of India** organized by Agharkar Research Institute, Pune, Maharashtra. 6-7 February 2003.
3. National Science Symposium on **Environmental Science and Technology organized** by Commission for Scientific and Technical Terminology and Community Science Center, Rajkot, Gujarat. 6-7 January 2003. (In Hindi)
4. National Conference on **Environmental Biology** organized by Department of Biosciences, Saurashtra University, Rajkot, Gujarat. 17-18 October 2002.
5. State level seminar on **Frontiers and Advancements in Biological Science in New Millennium** organized by Shree M. & N. Virani Science College, Rajkot, Gujarat. 11 August 2002.
6. National Conference on **Fungal Diversity and Biotechnology** organized by Mycological Society of India, Thane, Maharashtra. 2 – 4 February 2002
7. Workshop on **Principles and Methods in Regulatory Toxicology** organized by Jai Research Foundation, Vapi, Gujarat. 28 – 31 May 2001.